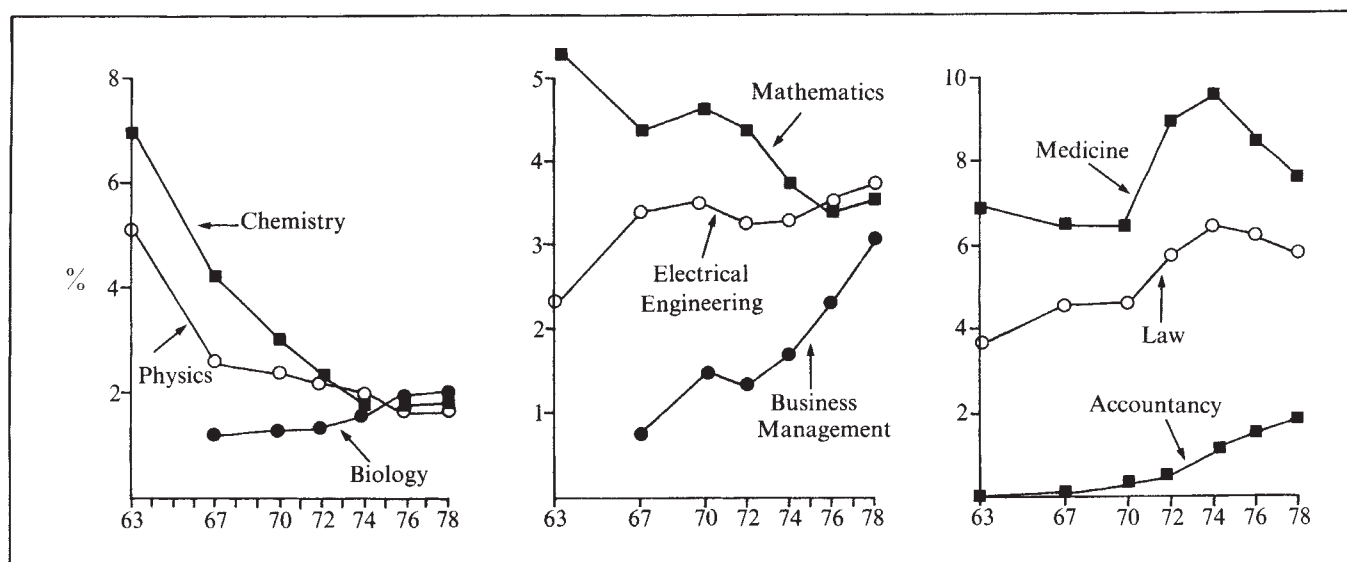


nature

19 October 1978

Popular science



THE above three graphs show changing patterns in popularity of various subjects amongst applicants to British universities over a period of fifteen years. The figures have been compiled from annual reports of the Universities Central Council on Admissions (UCCA), culminating in the Statistical Supplement to the Fifteenth Report, published recently. These reports have over the years consistently listed the number of candidates applying through the UCCA scheme for entry to any British university (excluding the Open University, University College, Buckingham and to a lesser extent three Scottish universities) by their first choice for study.

There were some changes in subject headings between 1963 and 1967 which mean that comparisons must be made a little cautiously (although there seems no evidence that the subject headings are seriously incompatible). Since 1967 the headings have remained the same, however, so there is no reason to doubt that year-by-year comparisons can be made. The figures are percentages of all applicants naming particular subjects as first choice. The total number of applicants in 1963 was 51,596 and in 1978 is about 180,000. Between 1963 and 1967 the number rose to 90,952; it has risen more slowly since then. Only one subject, chemistry, actually has less applicants in 1978 than in 1963.

As with all statistics, some cautions have to be given. These are not figures for admissions to university, so they do not reflect actual changes in size of intake. Very many students naming a preferred subject are happy, or at least willing, to be accepted for another subject. Furthermore, the figures say nothing about the quality of candidates. Many of those who apply simply do not have the necessary qualifications, and cannot be regarded in the final analysis as serious candidates for a university. Also amongst those who are serious candidates, there is probably substantial variation from subject to subject in the quality of applicants. Finally, the figures for 1978 are only estimates, though accurate ones.

What can be claimed is that the graphs represent fairly well the ambitions of 17-year-olds over a period in which a much greater number of students have been able to contemplate university education, and in which society's attitudes to various professions have changed substantially. Perhaps there are two points worth underlining. The first is that the vast expansion in educational opportunities in the 1960s did not stimulate in any way a proportional increase in the number of those doing the 'central' sciences. Secondly, contrary to popular belief, enthusiasm for engineering courses has actually grown fairly steadily for many years. □



Power-failure knocks out Seasat

A POWER-FAILURE thought to have been caused by a short circuit has cut off all communication with the experimental ocean-monitoring satellite Seasat-A, launched less than four months ago by the National Aeronautics and Space Administration.

Attempts by NASA's Jet Propulsion Laboratory in California to re-establish contact with the satellite have so far been unsuccessful. And it is feared that the satellite, which cost \$75 million to put into orbit, and was expected to continue to provide data for up to three years, may now have to be written off.

Seasat is primarily a "proof of concept" satellite designed to test the performance of a set of microwave sensors, including in particular a synthetic aperture radar which is able to gather data on the behaviour of the ocean's surface even through dense cloud.

In the four months since the satellite was launched considerable quantities of data have been successfully gathered and stored for future processing. NASA officials claim that the results processed so far indicate that the behaviour of the equipment has been "amazingly good".

However the apparently short life of the satellite has been a major disappointment to scientists who had expected to make use of a full range of data in a series of oceanographic and meteorological studies not only in the US, but also in Canada and Europe.

Canada's Department of Energy and Mines, for example, which is collecting Seasat data at a receiving station in Newfoundland, was in the process of a major survey of arctic

ice flows using simultaneous data from aircraft and the satellite. The survey will now have to be completed using aircraft data alone; and although the impact on the project is not likely to be substantial, one scientist described the loss of further Seasat data as a "considerable blow".

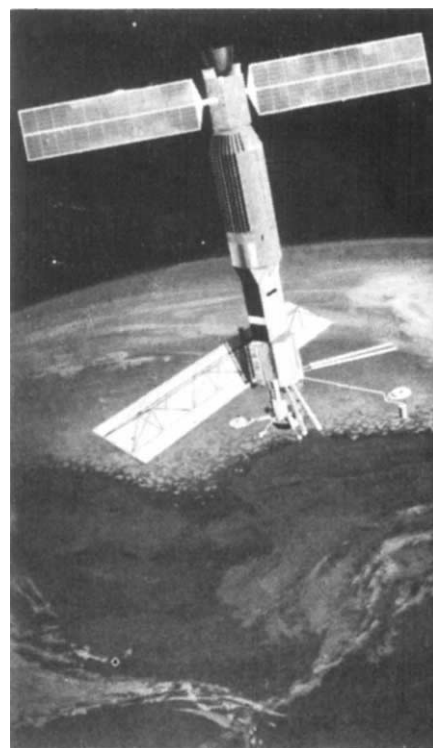
The trouble occurred last Monday when Seasat was in its 1503rd orbit. According to NASA officials the satellite seemed to be behaving normally when it passed over the receiving station at Orrora in Australia; however when it came within sight of the next station at Santiago in Chile, it was discovered that the batteries were discharging at full rate.

As the voltage dropped rapidly, various sensors began to shut down, and within a few minutes the satellite ceased to respond to commands sent up from ground control stations. A little later communication from the satellite to Earth also cut out.

Laser beams bounced off special reflectors on the bottom of the satellite as it passed over Bermuda showed that it was still on course. However continued attempts to get the satellite to switch off all non-essential equipment, in the hope that solar cells might recharge the batteries, have apparently so far met with little success.

Various bursts of data thought to be from Seasat were reported last week by several receiving stations, including one at Oakhanger near London, but it turned out that these had been transmitted from the earth-monitoring satellite Landsat-3.

The cause of the power failure is not yet known (although NASA officials were denying rumours that it might



have been knocked out by a Soviet anti-satellite weapon). However late last week it was discovered that a recorder left on for practice purposes at Oakhanger had been receiving signals at the time of the failure, and its data is now being studied to see if any clues to the short-circuit could be found.

Because of tight budget restrictions, Seasat-A is a one-off satellite with no back-up available. Although officially planned to remain in orbit for only one year, it had been supplied with sufficient resources to last for three. The soonest that a follow-up satellite could be launched, even with Congressional approval, would be 1983. □

Carter outlines future strategy for space science

PRESIDENT Carter has officially confirmed that there are unlikely to be any further US "space spectacles" such as the Apollo project during the next decade, and that while the administration remains committed to a strong space science programme, this must remain sufficiently flexible to absorb financial constraints "when conditions warrant".

However, included among future space science goals will, in addition to planetary studies currently underway, be more detailed exploration of Saturn and its moons and rings, and the reconnaissance of comets and asteroids.

These decisions were announced last week following a policy review of civilian space policy carried out by an

interagency committee set up four months ago under the chairmanship of Dr Frank Press, director of the Office of Science and Technology Policy.

The president's statement contains few surprises, and reflects the more cautious—some would say conservative—path that the National Aeronautics and Space Administration has been pursuing in the past few years. Its general message is that if there is to be a major expansion in space activities, the main stimulus is to come from private industry rather than the federal government.

As for space science, the statement lays down broad principles rather than specific objectives. Thus President Carter says that US space policy will

reflect a balanced strategy of application, science and technology development which will "assure American scientific and technological leadership in space for the security and welfare of the nation".

Such a policy will, he says, "emphasise space science and exploration in a manner that retains the challenge and excitements and permits the nation to retain the vitality of its space technology base, yet provides short-term flexibility to impose fiscal constraints when conditions warrant."

The president's statement says that it is neither feasible nor necessary at this time to commit the United States to a "high-challenge space engineering initiative" comparable to Apollo, and

that the now declining costs of the Space Shuttle development programme gives the administration "the flexibility to give greater attention to new space applications and exploration, continue programs at present levels, or contract them." Funds to cover these will be "adequate."

Turning to space science, the president says that in addition to continuing a vigorous programme of planetary explorations to understand the origin and evolution of the solar system, the US will utilise the space telescope and free-flying satellites to usher in "a new era of astronomy" which will include the exploration of interstellar molecules, quasars, pulsars and black holes.

The most controversial part of the statement comes in the section covering space applications, and in particular the future direction of earth monitoring systems such as LANDSAT. The

administration has been under Congressional pressure for some time to come up with a clear set of policy goals and long-term objectives.

Last month, for example, Senator Adlai Stevenson, chairman of the Senate subcommittee on science and space, introduced a bill which would require the president to present Congress annually with a five year forward-plan clearly laying out the administration's space objectives and how it proposed to meet them.

However, so far the administration has backed away from any firm commitment of this nature. And last week's statement merely says that, in the case of remote sensing systems, NASA will chair an interagency task force "to examine options" for an integrated system, and that a comprehensive plan covering all aspects of remote sensing "will be explored." □

World climate conference thrown open to social scientists

WHEN the World Climate Conference meets in Geneva in February next, it will involve some 400 participants instead of the 100 or so originally envisaged by its organisers at the World Meteorological Organisation (WMO). So now the social as well as the physical sciences can be represented—a move in line with the new trend of thinking on the role of science in the United Nations system. This broader participation also underlines the main objective of the conference "to examine the impact of climate on various aspects of human welfare, and especially of climatic fluctuations on all time scales".

WMO's Secretary General Dr D. A. Davies points out that the effects of cold spells and droughts have in recent years presented grave problems affecting energy supplies and agricultural output even in the most highly developed countries. At the other end of the economic ladder, the effects of the disaster caused originally by the drought in the Sahel in the early 1970s

are still causing grave concern. So this effort to bring about a "meeting of the minds" between the meteorologists and the behavioural scientists, biologists, economists and others in relevant disciplines is certainly not being made a moment too soon.

Nevertheless no great scientific surprises are expected to emerge from next February's meeting. As WMO sees it, hard evidence does little to support many of the disaster hypotheses of the doom-watchers. It is admitted, however, that there is one major exception: the problem of increasing CO₂ in the atmosphere. This is something in which WMO is seriously engaged, and is considered important enough for a major global effort to be made in the immediate future: the proposal is to use a wide network of regional observing stations, together with a smaller number of permanent baseline stations sited so as to cover the whole of the Earth's surface.

Peter Collins

"Erosion of spirit" in British science

THE morale of academic research scientists in British universities is at a low ebb, according to the Trustees of the Nuffield Foundation, a British charitable organisation which supports university research.

Writing in the annual report of the foundation, the trustees claim to observe "what appears to be an erosion of the spirit in scientific and medical research at British universities and polytechnics". The trustees base this

assertion on the fact that the Nuffield Foundation "has received many fewer applications of high quality for research grants in science and medicine than would have been expected if university departments were bursting with ideas and needing only financial resources to carry them through". The morale of the younger members of the community is particularly affected, says the report.

The trustees select lack of growth in

ICSU investigates refused visas

ICSU the International Council of Scientific Unions—is to investigate the complaint of a group of Israeli geneticists who failed to receive visas for the fourteenth International Congress of Genetics, held in Moscow last August.

Originally, 33 members of the Israeli Society of Genetics registered for the congress and received a registration number, indicating that their application was accepted. However, a month before the congress, visas had not yet arrived.

On 10 August, the Israeli Society of Genetics cabled Professor Ralph Riley, secretary of the International Genetics Federation, suggesting that the federation withdraw its sponsorship of the congress. This, it appeared, was impossible, both because the federation was financially committed, and also because it was feared such an action would "throw Russian genetics into turmoil again".

Eventually 18 visas arrived — restricted to the exact dates and place of the congress and with no provision for accompanying spouses nor for participation in the traditional post-congress excursions. At this the Israeli Society of Genetics decided to recommend its members not to attend—a recommendation which was duly followed. Instead, two delegates attended to put the Israeli case formally.

After various unofficial discussions, the case was raised at the representative council of the federation, and a resolution was passed, mild in tone, but demanding that "steps should be taken to ensure that all applicants to further congresses receive their visas in due time".

Last month, when the ICSU general assembly met in Athens, the resolution was brought to the attention of the Committee for the Free Circulation of Scientists. The Committee, which includes among its membership a representative of the Soviet Academy of Sciences, has undertaken to look into the matter.

Vera Rich

the universities as the main culprit: "younger people . . . can no longer set their sights on what until quite recently was the chief objective of young creative scientists—to be so successful in their chosen field as to make a mark not only in the scientific literature but on the pattern of research in their institutions."

The foundation offers to help—if anyone can come up with a practical proposal. □

Brazil's scientists fan doubts over energy priorities

David Dickson describes how problems with Brazil's nuclear programme have highlighted tensions between the government and the scientific community

ONE of the major issues that Brazil's new president will have to face when he takes power after next month's elections is whether the country should make a major shift in its energy strategy.

Officially the central component of this strategy remains a firm commitment to the rapid development of nuclear power, a programme under which the country is scheduled to possess nine power stations by 1990, and to be meeting 40% of its energy needs with nuclear power by the year 2000.

Criticism of the programme by Brazil's scientific community on technical, economic and political grounds appears so far to have been virtually ignored by the country's military leaders. And external criticism, such as the US's attempts to dissuade Germany from selling Brazil a re-processing plant on the basis that it would increase the danger of the proliferation of nuclear weapons, has hardened the nation's resolve to pursue its nuclear goal.

However, three factors are now putting strong pressure on Brazil's leadership to revise its current stance. The first involves problems and delays that have already occurred in the nuclear programme itself; in particular the extra construction work that has been made necessary by the discovery of geologic instabilities at present reactor sites.

The second is the rapidly increasing costs of the programme. Partly a result of the costs of the extra design and construction work, and partly due to the declining value of the cruzeiro against the Deutschmark, nuclear

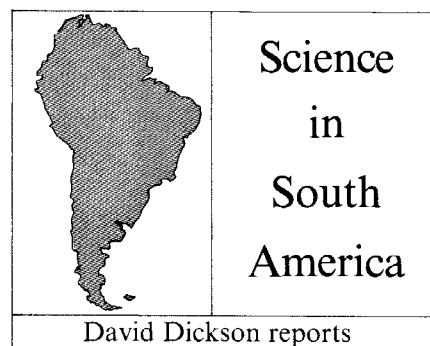
power is becoming a much less attractive proposition on economic grounds when compared to more conventional sources such as hydropower.

The third factor is the success that has already been achieved in Brazil's crash programme to substitute the use of ethyl alcohol for petroleum. Unlike nuclear power, the alcohol programme is well on the way to meeting its initial target of 4 billion litres a year by 1980; a further source of pride is that it has been based almost entirely on indigenous research and development. In many of Brazil's major cities cars now use a mixture of 20% alcohol, and by 1981 one-sixth of all new cars are planned to run on alcohol alone.

At the very least, some of Brazil's scientists now argue, the \$1.6 billion alcohol programme has removed the worst pressures of the energy crisis which had had such a dampening effect on Brazil's previously explosive economic growth, thus giving the country more time to consider the details of its nuclear future.

"Most scientists and industrialists would probably prefer a more modest programme in which they could play a dominant role and acquire the necessary technology" according to Professor Jose Goldemberg, director of Sao Paulo University's Institute of Physics, who has suggested that a prototype reactor working on the thorium cycle might be the most appropriate way forward.

Others go further to suggest that, given the vast potential of the nation's renewable energy sources—in particular in the fields of hydropower, solar



energy and the use of biomass—the most desirable option from both a technical and a social point of view would be for Brazil to drop its nuclear programme altogether.

The oil crisis of 1973 hit the country particularly hard. Unlike neighbouring Venezuela, it has little petroleum reserves, and intensive prospecting has been relatively unfruitful; 83% of its petroleum is now imported, at an annual cost of \$4 billion.

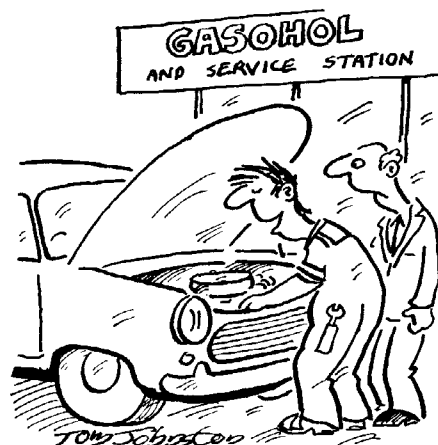
Even before the crisis, Brazil had committed itself to nuclear energy, signing an agreement with the US firm Westinghouse to build a 625 megawatt reactor on the coast at Angra dos Reis, 115 miles south-west of Rio de Janeiro, in 1969.

Construction work started in 1972, but has been plagued with problems. These range from an embarrassing fire last year, which caused \$10 million of damage to equipment and raised awkward questions about construction procedures and security, to the discovery that the site was on top of a rock platform that was slowly being pushed into the sea—and that a retaining wall was therefore needed to shield it from the pressure of earth moving down a nearby mountainside.

Other problems have been experienced with the subsequent contract for the construction of eight 1,300 megawatt reactors which Brazil signed with



Cars in Sao Paulo now run on a mixture of 20% alcohol



It's all right, sir. It's only suffering from a hangover!



Plagued with problems: Brazil's first nuclear power station under construction

West Germany in 1975. The package included not only equipment, materials and technical assistance, but also covered the construction of an enrichment plant and a reprocessing plant, providing, it had been hoped, the basis for Brazil's nuclear power programme to become self-sufficient.

It has been the inclusion of the enrichment and the reprocessing plants that have so far attracted the most outside attention. In addition to the Carter administration's attempts to block the sale of the latter, the Dutch government almost refused to allow the supply of enriched fuel from the European Urenco consortium because of concern over safety arrangements.

Such arguments, however, have few supporters inside Brazil. The military rulers have accused the US of interfering in another country's sovereignty, the same reason given for refusing to sign the Non-Proliferation Treaty; and even the government's political opponents, while expressing concern on the safety issue, have rallied behind it in rejecting outside pressure as "technological imperialism."

However, criticism of other aspects of the programme has been far from absent. Three issues in particular have attracted the attention of Brazil's scientific community, among whom the physicists have been especially vocal.

The first criticism is that, in buying the technology as a total package with no alteration permitted to the German design—even though the construction of parts will slowly be taken over by Brazilian contractors—no real transfer of technology will have taken place, and the Brazilians will have learned little about reactor design.

"The technological aspect of nuclear energy is vitally important, and we would like to learn how to develop our own technology so that we can develop a truly independent programme," says Professor Jose Moreira of the Institute of Physics, who claims that the short period spent in Germany by most of those sent to learn about nuclear tech-

nology means that little transfer of knowledge is likely to take place.

The second criticism relates to the proposed method for enriching uranium. The Germans have proposed using the as-yet unproven Becker jet-nozzle process. Research results, however, have so far been disappointing, and have led to the crack that Brazil is paying with money it does not have for technology that Germany does not possess.

Both of these relate to the third, broader criticism; that in developing its nuclear programme, the Brazilian government has taken little account of what its own scientists could or might be able to offer, and has thus neglected the opportunity to build a nuclear power industry truly independent of foreign interests.

Professor Moreira points out, for example, that at present Brazil has about 800 PhDs in nuclear physics, more than enough to establish a viable reactor research community, but that universities and technical institutes have been virtually ignored in the government's nuclear plans.

Similarly a group of physicists recently produced a report on "Panorama and Perspectives on physics in Brazil", which criticised the lack of interaction between the government nuclear company Nuclebras and the physics community, claiming that this appeared to be a deliberate policy, and pointing out that the considerable scientific resources of Nuclebras were not available to outside scientists.

The situation has historical roots. Back in the late 1960s when the nuclear plans were being drawn up, universities were a focus of opposition to the military government's authoritarian policies, and many staff members—including scientists such as Dr Leite Lopes, then scientific director of the Brazilian Centre for Research in Physics, and now professor of particle physics theory at the University of Strasbourg—lost their jobs.

A reluctance at the time to provide university research funds for a subject

as sensitive as nuclear energy is therefore little surprising. And the government even disbanded the main research group in the country, the Institute of Radioactive Research at the Federal University of Minas Gerais.

One consequence of the lack of involvement of universities, however, has been the reported difficulty of attracting scientists of sufficient calibre into the nuclear programme. For example it is claimed—although difficult to confirm, since the whole programme is shrouded in secrecy—that this is one reason why government laboratories have had such difficulty in developing the enrichment technique, even on an experimental basis.

"More than the petrol crisis, what is obstructing Brazilian development is the lack of human resources," Professor Jose Ellis Ripe, a physicist from the University of Campinas, told a conference last month on physics education. This, he said, was the result both of a low investment in education, including in particular education in the basic sciences, and a preference for investing government money in areas that promised a quick return.

One area of slightly less sophisticated technology in which Brazilian scientists and technologists have been able to get a firm grip has been in electric power transmission. Brazil and Paraguay are currently building what will be the largest hydroelectric dam in the world on the Parana river, eventually producing 12.6 gigawatts; Brazil has agreed to buy any power that Paraguay does not need, but since the two countries' electricity supplies are at 50 and 60 cycles a second respectively, Brazil is transmitting the power to its urban centres as direct current before reconverting it to alternating current.

Factors such as the need for smaller cables means that high voltage direct current transmission becomes more economical over a distance of about 700 kilometers. And engineers are now putting together ambitious plans to use such techniques to transmit hydro-power 2000 kilometres from the Amazon basin to the country's industrialised areas in the south, whose energy needs had previously stimulated the nuclear power programme.

But the area which has given the greatest boost to Brazil's self-confidence in its research capabilities has been the alcohol programme. From modest beginnings only a few years ago, the research programme has mushroomed under the personal support of President Geisel to a position in which several hundred separate projects are planned or operating, with a total budget of \$65 million over the next two years.

Even US scientists admit that this is an area of energy research and deve-

lopment in which Brazil has established an advantage over all other countries, and there has been much international interest—for example from scientists in West Germany and Scandinavia—in the programme and its results.

Reception of "gasohol" by consumers has been mixed; there are complaints of timing problems—the streets of São Paulo are filled with frequent back-fires—of difficulties in starting on cold mornings (overcome by the addition of a small pre-heater) and of an increase in fuel consumption.

Research scientists at the Aerospace Technical Center near São Paulo, where much of the enthusiasm initially came from and where there are currently 34 separate programmes looking at engine design, insist that such problems are relatively minor, and that the technical feasibility of using alcohol in petrol on a large scale has now been clearly established.

In other places, much research is being carried out into the supply side. Initially the prime candidate for transformation into alcohol had been manioc, a root vegetable widely used by Brazil's native Indian which grows in poor soil with relatively little water—two characteristics of Brazil's Northeast region in which social conditions are worst, and needs are greatest.

However at present the main emphasis is being placed on methods of producing alcohol from sugar cane. Although unlike manioc, which can be harvested at any time, cane can only be collected for six months of the year, it is considerably easier to extract the sugar required for fermentation, and the outside sheath, known as bagaco, can also be burnt to provide the energy for the fermentation process.

Government officials, their appetite whetted by the apparent success of the alcohol programme, are now busy following up the potential uses of other biological materials. One prime candidate under consideration is the nut of babacu palm tree, at present used almost exclusively for its seeds, which are extracted from the nut's core and crushed to produce their oil.

Plans are now being studied to use each of the various parts of the nut for different purposes; the seeds to make palm nut oil, the core to make charcoal and gas, the shell to make alcohol, and the skin for primary combustion.

In addition to building the self-confidence of the research community, the whole field of alternative energy sources is proving to be a fruitful outlet for university scientists who had been previously frustrated in attempts to link their work to socially useful objectives.

The involvement which started with criticism of the government's nuclear

policy is now being channelled by some into more pragmatic goals. This process has been encouraged by the availability of funds from the federal government, which has recently set up an office in the Ministry of Mines and Energy, under the title of Project Ipiranga, to centralise research efforts on non-conventional sources of energy.

A group of physicists at the Federal University of Rio de Janeiro, for example, under the direction of Dr Luiz Pinguelle, is now looking at the energy options facing state power companies.

Similarly Professor Goldemberg and Professor Moreira in São Paulo are looking at ways of increasing the efficiency of the conversion processes applied to biological materials. In particular, they are investigating both theoretically and experimentally ways of using fluidised bed combustion to increase the efficiency of burning the sugar-cane bagaco which they claim could be used as an important source of electricity for other domestic and industrial purposes.

But the more general sense of frustration and exclusion remains. Many scientists, for example, are now calling on the government to permit the return of those of their colleagues who had previously been expelled, claiming that the country needs to do what it can do build a base that can shore up its ailing economy.

There have also been requests for greater participation in decision making on technological issues, as well as in the assessment of the general direction of bodies such as the National Research Council, Nuclebras, and even the Ministry of Education.

So far, despite the slight loosening of earlier restrictions, there has been little response to such requests. And in energy policy at least, there remains the feeling that political interests continue to play a dominant role.

One reason for the switch from

manioc to sugar cane in the alcohol programme, for example, was that despite the social advantages of the former in bringing work and income to the country's most depressed areas, the dramatic decline in world sugar prices in 1973/74 meant that the large sugar-cane producers needed new outlets for their produce, and pushed for their inclusion in the programme.

Nor are established interests far below the surface in the nuclear energy field. Thus there has recently been a scandal involving the close connections between the Minister of Trade and Industry and the owner of a previously obscure engineering company which was awarded, without any competitive bidding, the contract for the construction work for the first two German reactors, Angra 2 and 3.

Furthermore Brazil can expect heavy pressure from Germany if its attempts to back out—as Iran is now reported to be contemplating—from its previous commitment, even though, according to Mr Arnaldo Barbalho, president of the state-owned electricity company Electrobras, "the agreement speaks only of the purchase of up to eight nuclear power stations."

Finally with Argentina, Brazil's main economic competitor in the South American continent, already successfully operating one reactor and building more, any suggestion of cutting back on nuclear plans is unlikely to be warmly received in military circles.

Last month the Brazilian congress, largely under pressure from the opposition party MDB, agreed to set up in the Senate a parliamentary commission of inquiry into the country's nuclear policy. Some scientists feel this could be an opportunity to explore the viability of a major shift towards alternative, mainly renewable energy sources. The technical potential has been demonstrated; the political will is now required. □

Argentina plans nuclear reprocessing plant

ARGENTINA is soon to start construction work on an experimental plutonium reprocessing plant, and could be operating a complete nuclear fuel cycle with domestic technology by 1990, according to Admiral Raul Castro Madero, President of the Atomic Energy Commission.

The reprocessing plant will be situated in the grounds of the Ezeiza Atomic Centre outside Buenos Aires. A laboratory scale reprocessing plant was built at Ezeiza in the early 1960s, and is said to have produced a few milligrams of plutonium before it was dismantled.

According to a report in the

Washington Post, Argentina is also planning to start production of a heavy water plant. The country already operates a natural uranium reactor—the only nuclear reactor presently operating in South America—and this would give it the capacity to produce nuclear fuel using domestic technology.

Since Argentina has not signed the nuclear non-proliferation treaty, it would be able to produce weapons-grade plutonium free of international safeguards. However, Admiral Madero said in an interview that the country had "no need for nuclear weapons". □

Israel's place in the sun



Israel's abundance of sun and scientists has led to great interest in the application of solar energy, writes **Vera Rich**

ISRAEL, it may be claimed, has had an interest in solar power dating back some 3,500 years—when Joshua ordered the Sun to stand still! But today there is a boom in the more scientific uses of solar energy in the country.

The modern era of Israeli solar power dates back to 1911, when a mining engineer from Siberia, Moshe Novomeiskii, first visited the Dead Sea. Novomeiskii realised that the combination of arid atmosphere and virtually unlimited sunlight (the annual rainfall is about 5 cm) would make the extraction of mineral salts by evaporation commercial. By 1930, the first plant, at the northern end of the Dead Sea, was in operation, and in 1934, a second plant was opened at S'dom, at the southern end of the sea, where the terrain is suitable for the construction of very large evaporation pans.

Following partition in 1948, the northern plant came into Jordanian hands, and was later destroyed; but the S'dom plant has become the basis of Israel's potash and chemical industry. The evaporation pan system now covers 130 km² and has a capacity sufficient to produce 1,250,000 to 1,400,000 tons of phosphate a year, much of which is exported, as well as bromine compounds and magnesium and sodium chlorides. The solar energy consumed in the evaporation process is estimated to be equivalent to more than Israel's annual output of electricity.

For some years, the Dead Sea works remained Israel's only effort in the use of solar energy. Then, about 20 years ago, the first solar-powered hot-water systems began to go into operation. At

the present time, even the prefabricated homes of the small Negev settlements have roof-top water heaters (two solar panels and a drum), and it is estimated that some 35% of the population enjoys solar hot-water—a total which would doubtless be higher had not certain local authorities banned roof-top storage tanks for aesthetic reasons.

Following the oil crisis of the early 1970s, and in view of the fact that Israel has so far located only minor deposits of oil and gas within the "green line" (the pre-1967 frontier), there is now a boom in all types of solar energy exploitation. At virtually every institute of higher education in Israel, a number of solar energy projects are going forward, in any available corner. Even the spacious Weizmann Institute seems to find it impossible to house all its solar team in an appropriate building, and one can find solar energy work going on, for example, in the basement of the animal breeding centre!

Broadly speaking, solar energy research falls into four main sections:

- passive domestic systems: water heaters, space heating and air conditioning;
- production of electricity from solar energy;
- use of photosynthesis and related processes to "fix" solar energy, and
- miscellaneous applications.

Research into passive domestic systems is now well advanced, and ranges from the development (at the S'de Boquer Desert Research Centre) of lightweight collector panels which the householder can install himself, to detailed investigations of the technical and economic problems of central

heating systems for apartment houses and other large establishments. Such a survey was carried out at the Ben Gurion University, Beer-Sheva, using the students' hostels as a pilot system.

A number of heating/air conditioning systems are being studied. At S'de Boquer, a whole line of one-room 'houses' has been erected, to permit the variation and comparison of the structural parameters involved. The underlying concept of the S'de Boquer research is that the development of the Negev should guarantee a reasonable quality of life for those who settle there; comfortable and appropriate housing is therefore all-important.

The storage element in these systems is simply a rough-fill of local stones and pebbles, which considerably reduces the cost. A somewhat similar principle has been tried at the Government Agricultural Research Station at Vulcani, for the heating of chicken-houses, but with less success; largely because chicken-house heating requires a series of peaks, rather than a steady background heat, and it is difficult to work out an economic regime of operation. Similarly, solar drying of tobacco failed to give the right leaf colour. The Vulcani team are not, however, too disappointed; the research has taught them a good deal, they consider, in the management of solar energy, and the solar drying technique might yet prove commercially viable for herbs such as oregano and mint.

Active solar energy systems, producing electricity from solar power, present a more complex problem. Economically speaking, the main problem is the cost of the silicon cells. In the USA, investments of the order of several hundred million dollars have been made in attempts to reduce the cost of the cells, and have succeeded, over the last two years, in reducing the effective cost of solar-cell electricity from \$40 to some \$12/Watt.

At the Weizmann Institute, however, a different approach is used—high-concentration arrays, using plastic Fresnel lenses to concentrate the sunlight onto small, relatively low-cost cells, and a mechanical tracking system to keep the array directed towards the Sun.

Over the last five years, considerable efforts have been made to incorporate the latest technological developments into the fabrication of high-efficiency solar cells, so that, at present, arrays of the Weizmann type can compete with small diesel generators. In the opinion of Dr Yosef Mandelkorn, by 1985, the cost of high-concentration silicon arrays will be low enough to warrant their use in self-contained urban communities away from the main centres of population.

One very interesting development

Figure (above): a large on-line Sun-tracking system for solar energy experiments at the Weizmann Institute of Science, Rehovot. The system can drive 2 m² of solar concentrators

of this kind of array, he added, is the possibility of using solar cells for the simultaneous supply of electrical and thermal (hot-water) energy, by mounting the cells on a structure of pipes through which a heat-exchange liquid flows.

In Dr Mandelkorn's opinion, use of high-concentration arrays could reduce the cost of silicon cell power generation to \$2 to \$4/Watt in the very near future—a figure which most experts would, he said, consider "realistic".

A far more radical approach to solar power generation is that of Dr Branover of the Ben Gurion University, Beer-Sheva. His project, which is supported by the Israeli Ministry of Energy, uses solar power to heat a low-boiling point liquid to about 70 to 80 °C, so that its vapour mixes with a suitable liquid metal (mercury, gallium, Wurtz metal) and accelerates it to a velocity of some 30 to 40 m/sec.

At this velocity, the liquid enters the field of a permanent magnet (about 0.5 Tesla). Depending on the circuit parameters, with this velocity and field it is possible to generate a field of about 1V with a current strength of up to 1,000 A.

Low temperature magnetohydrodynamic (MHD) generation of this kind, says Dr Branover, offers many advantages; it has no moving parts save the liquid metal, and is hermetically closed once for all. By connecting several generators in series, it is hoped to produce direct current of around 1,000 A at 10 V.

Such a power source, says Dr Branover, could be used in the chemical industry for producing copper, magnesium and aluminium. It could also be used for charging batteries for electric cars, which could change their batteries at garages in the same way that they now fill up with petrol. This would facilitate the change over from petrol-driven to electric automobiles without disrupting either the infrastructure of garages nor the fiscal system (since governments could simply tax the batteries instead of the petrol); however, this, says Dr Branover, would not be practical before about 1990, since the onus is on the automobile

manufacturers to produce a suitable vehicle.

His low-temperature MHD plant should, however, be in operation, at least at the pilot stage, by 1982; all the preliminary calculations are done, and work is now going ahead on building the model.

Another imaginative use of solar energy as a power source is based on *Dunaliella*—the only life-form capable of surviving in the Dead Sea. This alga manages to survive the high osmotic pressure by producing glycerol, which can amount to 85% of its dry weight. Possibilities of the commercial production of *Dunaliella* are being worked out both by the Weizmann Institute and the H. Steinitz Marine Biology Laboratory (Eilat) of the Hebrew University. The main problem would appear to be the harvesting of the alga; possible centrifuge methods are under investigation.

Algae production as a means of fixing solar energy is a popular subject in Israel, which hosted an international conference on the subject at Acco last September. One of the most imaginative schemes, worked out by a team from the Technion, headed by Professor Gedaliahu Shelef, uses photosynthesis, bacterial action and algae growth to purify sewage by a process of aerobic digestion accelerated by aeration and flotation techniques. The algae is recovered and used as high-protein animal feed, and the recovered effluent can be used for irrigation. A pilot plant is already in operation in Haifa; introduction of the process on a commercial scale, says Professor Shelef, could save the Israeli economy the several million dollars a year presently spent on the import of soybeans and fishmeal.

The use of essentially natural processes as energy sources seems to have a particular appeal to Israelis, both from an economic point of view, and also, as was frequently pointed out at the Intecol conference in Jerusalem last September, Israel is geographically too small a country to carry an ecological disaster or major pollution.

One such process, which is now being implemented on a pilot scale in

the Negev, is the solar pond. The original "solar lake" was discovered in 1967 in the Sinai, just south of the former frontier. It is a brackish water lake, with high salinity caused by evaporation, into which, during the winter, a certain quantity of less saline water seeps, forming a top layer. Since the lower layers are so dense, no convective mixing can occur, and accordingly, under the Sun's heat the bottom waters can reach a temperature of up to 60 °C. In an artificial solar pond, a suitable heat exchange system can be installed, and the heat extracted. The Weizmann Institute have even considered the possibility of using the Dead Sea itself as a solar lake. According to their calculations 50 square miles of the Sea would provide enough energy to double Israel's water resources by utilising the heat produced in a desalination process.

Perhaps, however, the most imaginative use of solar power is not as an energy source at all, but as a means of controlling soil-borne pathogens. In a process developed by Professor Ja'acov Katan of the Hebrew University, instead of using pesticides, with all their problems of side-effects on non-target organisms, biodegradation, and the like, the soil to be disinfected is simply covered in the summer by a sheet of plastic.

Field tests, taking samples from three layers of soil, showed that after 13 days the pathogens were killed even in the lowest layers. Block-randomised tests give dramatic evidence of the effectiveness of the method, which has already, incidentally, been adopted by some cotton farmers in the USA to control *fusarium*.

The main cost of the method is the plastic, which deteriorates in the strong sunlight and is therefore not reusable. Moreover, it is easy to apply—all the farmer has to do is to unroll the plastic and anchor it firmly.

It would therefore, says Professor Katan, be an ideal method to introduce in the third world, many countries of which share with Israel the prime requirement of any major solar energy programme—a hot climate and a clear sky. □

Red Sea muds ripe for exploitation

DEEP waters containing hot brine and metalliferous muds have been known to exist in the Red Sea since the mid-1960s. However, exploitation of this mud has been hindered by a mild dispute between Saudi Arabia and Sudan over Sovereignty of the Sea, by a lack of resources and by the absence of a suitable technology for mining it.

In May 1974 Saudi Arabia and Sudan signed an agreement on the joint exploitation of the metalliferous mud.

Under the agreement the Red Sea is divided into three zones: one extending westward from the Saudi coast to a line where the depth of water is continuously one thousand metres; one extending eastward from the Sudanese coast to a line where the depth of water is continuously one thousand metres; and a common zone lying between the two others. Each country has exclusive rights over the area between its coastline and the common zone; in the com-

mon zone both countries have equal and exclusive rights.

The agreement paved the way for exploiting the mineral wealth in the Red Sea. Almost exactly a year after the agreement was signed, the two sides established the Jeddah based Saudi-Sudanese Commission for the Development of Red Sea Resources. Each country is represented on the commission by three members; the head of each delegation being the

ministers of mineral resources. The commission is largely funded by the Saudis who hope to obtain a good return on their investment.

The commission is mainly interested in a 60 sq km area known as the Atlantis II deep. It lies midway between Jeddah and Port Sudan in the central trough of the Red Sea. It is here that the metalliferous muds are found. These muds contain 17 different metals in economic quantities ranging from zinc, silver, copper, cadmium, mercury, gold to a great deal of iron. The commission is undertaking extensive surveys and feasibility studies which are expected to be completed by 1981 at an overall cost of US \$100 million.

Before mining can begin a number of technical problems must be overcome. The Atlantis II deep cannot be mined with existing technology so the commission has had to develop its own technology for mining and processing the muds. The commission has done this with the Bureau de Recherches Géologiques et Minières of France, as technical consultants and with the assistance of the Arabian Geophysical Surveying Company, the Arab League Educational, Cultural and Scientific Organisation, the German mining company Preussag and the Universities of Hamburg and Duke.

Small mountains of the mud, which

consists of a superfine material exist in a highly saline, hot environment. The system developed to mine the mud involves agitating it to dilute it with sea water, and then sucking it up with a hydraulic pump. Special ship-borne flotation cells will collect a 5% concentrate of mud in brine. The concentrate will then be carried off to the shore for further treatment and metal extraction. The method of treatment and separation of the metals is claimed by the commission to be cost-effective with a recovery rate of 95%.

Pilot experiments on mining and the separation of metals have already been carried out and further experiments are planned. The mining system will soon be tested in the North Sea and the Mediterranean before final tests begin in the Red Sea. A Red Sea pilot test is planned for February next year.

Disposing of mining wastes has also presented a serious technical problem. Despite the increase in traffic through the Suez Canal, the Red Sea is one of the cleanest seas in the world. The commission is aware that its activities could be detrimental to the environment and sea life of the Red Sea.

The main environmental hazards will result from the diluted muds pumped up and the chemicals used in the flotation process being dumped back into the sea. It is expected that

the pollution would ultimately drift to those areas of the Red Sea that are rich in marine life. The commission is undertaking an extensive research programme, monitoring the environment and controlling the possible effects of this pollution.

The commission is aware that the mining and processing technology developed for the exploitation of the Red Sea, some aspects of pollution control technology, have been tailor made and may not be applicable elsewhere. Other Third World countries, however, can learn a great deal from the research and development activities of the commission. It has, therefore, established an information and documentation centre on the Red Sea. The centre is intended to be used primarily by Saudi and Sudanese researchers and scholars and as a facility for graduate studies. But it will also be open to researchers working on similar problems in other parts of the Third World.

Production is expected to begin around 1985, the yield being 100 thousand to 150 thousand tons per annum over the next 15 to 20 years. The present day value of located reserves in the Red Sea is US \$2.5–\$3 billion. The Saudis and the Sudanese hope to become almost self-sufficient in the minerals they extract from the Red Sea.

Ziauddin Sardar

Nuclear power reaches the Swedish stage

A LITTLE girl is playing a recorder by a river. A monstrous, jerking man closes in on her. She looks up, smiles. "Hullo", she says, "my name is Maria. Shall we play?" She picks flowers. They throw them into the river. She holds out her hands to him. He picks her up, and throws her into the river.

At the interval, two men approach me in the foyer. Each wears a T-shirt. On one T-shirt I read "nuclear reactor"; on the other, "nuclear weapons". As they go by, I see that they are joined by a tape which reads "nuclear waste".

The drama and the facts of nuclear power: nuclear reactors as Frankensteinian monsters, and nuclear reactors as objects of discussion and debate. This is the subject of the most controversial autumn theatre Stockholm has to offer: "The Storm" ("Stormen").

Originally billed for a nine-night run, but prolonged to more than twice that by public demand, "The Storm" is no ordinary production. Nearly all of the Royal Dramatic Theatre's 350 staff members have had a hand in designing the performance, and their enthusiasm is reflected in the energy-and-the-future exhibitions in the theatre's corridors, as well as in the blinking radiation signs, whirling wind vanes and solar panels sprouting from its stately roof.



Frankenstein: a nuclear reactor on the Stockholm stage.

The audience gathers in the main theatre at the beginning and the end of the evening, to see two hearings. The first of these is being held after an accident at a nuclear reactor. Who should take the responsibility for the accident? Various people are questioned. Their answers have been woven into a script from their organisation's actual official texts. The reactor's security chief insists that no accidents can happen; the man from Asea Atom (constructor of reactors) points out that his company only made the tur-

bine, which worked perfectly and had nothing to do with the accident; the lady from the Radiation Protection Institute insists that her institute is responsible for radiation protection only when the reactors are working properly; the State Power Board man can only repeat how much the country needs nuclear power for its balance of payments. The man in the control room at the time of the accident had not followed his instructions, and he is prepared to admit that he must take some of the blame. The message is clear:

the responsibility is nobody's; there is to be one sacrificial lamb.

The crux of the play comes with the second debate: can nuclear power and democracy co-exist? The hearing continues. More witnesses are called: politicians join technocrats. Arguments fly. Again, the message is clear: the complexity of nuclear technology puts it out of the public's control. Democracy has yielded to parliamentarism: the experts rule. But this can be changed if the people demand it. Technology, they should insist, must be intelligible to those who will be affected by it. The people should stand up and fight for a non-nuclear society.

Between hearings, the audience is escorted in small groups through mazes of corridors, past theatre staff notice boards, up and down old staircases and under backdrop stores to see some of the small plays being simultaneously performed in subterranean rehearsal rooms. The play I saw transported us to Baghdad, where a delegation from the International Atomic Energy Agency providing "atoms for peace" ended up supplying the Iraqis with their own atomic bomb.

Reactions to the play have only one thing in common: passion. "Possibly the Dramatic Theatre's most important cultural contribution ever" proclaims Hannes Alfvén, Nobel laureate and nuclear power opponent. "Monstrous lies—the threat to democracy is not nuclear power, but a cynical exploitation of the people's fear of it", declares Lars Lundgren of the Central Electricity Production Board (CDL). "Two things about the play annoyed me personally", adds Bo Kumlin (CDL). "One was a very anti-science feeling: the idea that science and technology as such are bad for the people. This is a very negative approach, which really has nothing to do with nuclear power. The other was that it purposely equated nuclear power with nuclear weapons, making it very hard for the audience . . . to know whether they were talking about the one or the other."

"We have been criticised for not trying to be objective", explains Jan Oqvist, employed as ideas man for the production. "But when the theatre staff started going to see nuclear reactors for themselves, they were shocked. They could not ignore the contradiction of the heavy security precautions, while the guides kept telling them that it was all perfectly safe and that nothing could go wrong. Then they were infuriated by the attitude of some of the scientists they talked to: 'Science is our preserve—what business have you amateurs got to play with it?' We had to impart our experience. The theatre can never be neutral."

Wendy Barnaby

Rocky road to funding



THOMAS H. JUKES

THE return of rocks from the moon by the Apollo expeditions, at considerable expense, gave the geological sciences a unique and unprecedented opportunity for extraterrestrial research with all its far-reaching implications. Biologists could admire the new findings, and try not to be envious. However, *Nature* reported on 10 August, page 522, that the continued funding of moon rock research had been completely blocked by a subcommittee headed by Senator William Proxmire.

It has seemed for some years that the Senator's reach for scientific matters exceeds his grasp of their significance. However, he is apparently alert to the vote-getting potential of issues including science. In 1974, he was successful in eliminating regulations that were designed to curb the expansion of the "health food" industry into ever-widening horizons of vitamin sales. (Incidentally, it seems strange that "health foods" need such heavy supplementation with vitamins.)

The FDA had proposed to restrain the excessive promotion of megavitamin usage by requiring the proponents thereof to establish its usefulness. The curb would have hit the health food stores in the pocket by reducing sales of high-potency vitamin products. The proposal did not affect vitamin pills of lower, but adequate content, each containing 1.5 times the recommended daily allowance. Any amount of vitamins can be obtained by swallowing large numbers of such pills.

Senator Proxmire led the fight in Congress against the regulations, and he introduced a bill to stop them. The bill was opposed by all major professional groups concerned with nutrition, who opined that the "Proxmire bill" catered to the health food in-

dustry and was contrary to the public welfare. It passed the Senate by a majority of 78 to 11, greatly aided by a spectacular letter-writing campaign that was mounted by the National Health Federation. This commercially based organisation sponsors laetrile and opposes fluoridation. One of Senator Proxmire's arguments was that the recommended daily allowances for vitamins had been set artificially too low by the Food and Nutrition Board of the National Academy of Sciences so as to benefit the food and pharmaceutical industries. He said that these industries had subverted the Board by paying its way. The argument seemed strange, because if the allowances were increased, the pharmaceutical industry would sell more vitamins, and the food industry would add increased amounts to foods.

The president of the Academy wrote Senator Proxmire a seven-page letter explaining that the Board was financed solely by a US Public Health Service grant. The letter was unanswered, and when I asked the Senator why, he told me that it was the typical response of a bureaucrat who had been caught in a conflict of interest.

The newspapers help Senator Proxmire by publishing what he calls his monthly "Golden Fleece" award for wasting tax dollars. The award frequently holds research proposals up to ridicule. This is an old-time headline getter. Years ago, a Secretary of Defense, Mr Charles Wilson, announced that his department did not intend supporting research on the question of why grass was green. Once a congressman made headlines by poking fun at an investigation of the "lovelife of cockroaches". This was good for a laugh, because it was well before the search started for pheromones to be used as replacements for insecticides.

Last month Mr Proxmire's target was a \$5,000 expenditure to exercise pregnant pigs on a treadmill. His witty comment on this, as reported by Reuters, was "The folks at U.S.D.A. went hog wild on this one. As a result, the taxpayers may get stuck with a pig in a poke."

To a taxpayer, the palatial Russell and Dirksen Buildings seem at first glance to be sufficiently commodious for the U.S. Senate. Better things are planned: a new, marble, \$135 million palace from which vantage point Senator Proxmire, if re-elected, will doubtless dispense more pearls of wisdom. Perhaps he will bestow a Golden Fleece award on the US Senate.

news and views

Incipient superfluidity in normal liquid ^3He

from P. V. E. McClintock

EXPERIMENTS by D. N. Paulson and J. C. Wheatley, described in a recent issue of *Physical Review Letters* (41, 561; 1978) provide the strongest evidence yet that liquid ^3He anticipates its superfluid transition. It seems that, when the liquid is cooled towards the transition temperature T_c , its properties first start to deviate from the behaviour characteristic of the normal (non-superfluid) liquid at a temperature which is significantly above T_c , almost as though the liquid somehow 'knows' that it is about to become superfluid.

At sufficiently low temperatures liquid ^3He does not support ordinary compressional soundwaves because the collision mean-free-path of the quasi-particles which would carry the wave becomes longer than the wavelength of the sound itself: instead, however, there is a peculiar collisionless form of wave propagation known as zero sound. It is this phenomenon which has been investigated by Paulson and Wheatley. Their experiment amounted, in effect, to a very careful study of zero sound and, in particular, of its attenuation coefficient α_0 close to T_c . The attenuation coefficient, which is defined by how rapidly the wave decreases in amplitude as it travels through the liquid, is known to depend accurately on the square of the temperature T when T is well above T_c ; but, at T_c itself α_0 starts to rise very rapidly, in fact almost discontinuously, as T is further reduced. What Paulson and Wheatley wished to ascertain was whether or not any departures from the T^2 dependence could be detected just above T_c .

Their experimental cell consisted of a pair of quartz transducers which were used, respectively, to excite and to detect the zero sound, thus enabling measurements to be made of the attenuation which occurred within the 12.7 mm path length of liquid ^3He which separated them. The cell was mounted in a nuclear demagnetisation cryostat capable of achieving and

maintaining the temperatures near 1 mK necessary to induce the superfluid transition (the exact value of T_c depending on the external pressure applied to the liquid). A particularly important feature of the experimental design, distinguishing it from some earlier zero sound cells, lay in the thermometry arrangements.

A paramagnetic susceptibility thermometer was used but, rather than the conventional compressed cylinder of powdered cerium magnesium nitrate (CMN) crystals, the authors used instead a cylinder of cerium lanthanum magnesium nitrate. By replacing 95% of the magnetic cerium ions by inert lanthanum ions, magnetic interactions

between the cerium ions were greatly decreased. This substantially reduced experimental uncertainties involved in converting from the so-called magnetic temperature T^* (defined on the assumption that the temperature is exactly inversely proportional to the measured susceptibility—which it is not) to the absolute Kelvin temperature T . To a good approximation, $T = T^* + \Delta$ where Δ is the required correction: for pure CMN, Δ is about 0.6 mK; whereas, for the diluted CMN used by Paulson and Wheatley, Δ was found to be 0.1 mK or less.

The importance of an accurate absolute temperature scale arose from the need to look for very small deviations from the T^2 law as T_c was approached: using pure CMN, a small error in Δ can easily produce the illusion of such deviations by introducing a systematic error in T . For the diluted CMN, on the other hand, Δ was already so small that the possibility of spurious effects of this nature could virtually be ignored.

Some experimental results are shown in Fig. 1. The zero sound attenuation α_0 divided by T^2 is plotted vertically, and the inverse of the temperature T is plotted horizontally. Both quantities have been normalised in such a way that they take the value unity at T_c . If there were no deviations from T^2 behaviour above T_c , the graph would follow the dashed horizontal line until the transition at $T_c/T = 1$ was reached, where it would then start to rise almost vertically. Clearly, the reality is far otherwise: there is a small but quite definite excess attenuation, starting well above T_c which grows increasingly rapidly as T_c is approached.

Comparable behaviour has also been seen, although not so clearly, in the static magnetisation (Paulson, Kojima & Wheatley, *Phys. Lett.* 47A, 457; 1974) and in the viscosity (Parpia, *et al. Phys. Rev. Lett.* 40, 565; 1978). In each case, the property seemed to start responding to the transition before T_c had quite been reached. The 'anomalies' were, however, a good deal smaller than in the case of zero sound, and they were hard to evaluate pre-

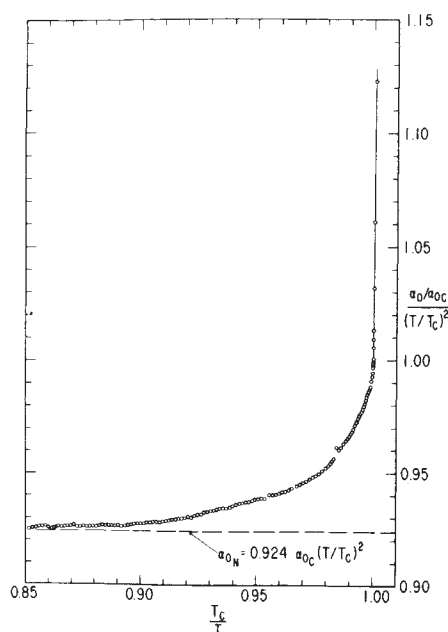


Fig. 1 The attenuation α_0 of zero sound in normal liquid ^3He divided by the square of the absolute temperature T , plotted as a function of T^{-1} . (Both ordinate and abscissa have been normalised in such a way that they are equal to unity at the superfluid transition, where $T = T_c$ and $\alpha = \alpha_0$). Departures of the experimental points from the horizontal dashed line indicate the extent to which the liquid anticipates its superfluid transition at temperatures above T_c . (After Paulson & Wheatley, *op. cit.*)

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cisely because of the thermometry problem referred to above.

The reason why liquid ^3He behaves in this way, apparently anticipating its transition, is probably associated with the existence of fluctuations: these may be pictured as the rapid transient formation and disappearance of tiny regions of superfluid, even when the liquid as a whole remains above T_0 . In the superfluid just below T_0 , α_0 is very much larger than for the normal phase, so that one consequence of such fluctuations might indeed be an 'anomal-

ously' large value of α_0 when T is just above T_0 . The magnetisation and viscosity change much less dramatically below T_0 , so that the effect of fluctuations on these properties just above T_0 would presumably be correspondingly smaller, in agreement with experiment.

There is, as yet, no quantitative theory to describe these phenomena, but it seems probable that strenuous efforts will now be made towards this end—particularly since studies of the corresponding effect in superconducting metals have proved so rewarding.

Conservation in Madagascar

from Alison Jolly

THE first international conference organised by Malagasy scientists on the problems of conserving their unique flora and fauna was held recently in Antananarivo.* The conference was convened to deal specifically with the Malagasy lemurs, a group of primates unique to the island. The government of the Malagasy Democratic Republic has postponed such discussion for the past five years for the good reason that conflict between shifting agriculture and forest conservation is one of the country's most intractable political problems.

Madagascar is one of the world's biogeographic regions. Of its roughly 715 vertebrate species, some 575 are endemic; so are at least 80% of its 10,000 flowering plants. Six families of plants, four of birds, and five of mammals are confined to Madagascar. Intact Malagasy rainforest, deciduous woodland, or spiny desert, where 90% of all the species are unique, show us Hutchinson's "ecological theatre and evolutionary play" with a different cast of characters.

Giving the keynote address, Joseph Andrianampianina, of the Directorate of Water, Forests and Soil Conservation, described lemur hunting, and habitat destruction by axe and fire: slash and burn clearings in the eastern rainforest, forest fires for grazing land in the dry west. When man reached Madagascar little more than a 1,000 years ago, the island-continent was wholly forested. The last full aerial survey, begun in 1949 and published in 1958, showed 20% forest cover. The American states from Maine south through Virginia, with the same area as Madagascar, have almost twice as much forest as Madagascar 20 years ago. The rate of clearance has accelerated to the point where the only

intact forests will soon be those defended by national action. In spite of the scientific importance of Malagasy wildlife, and its possible tourist value, Andrianampianina concluded, "We are perfectly aware that none of our suggestions, none of our practical advice, will achieve anything . . . for the protection of nature, if the people do not have land which they can cultivate in perpetuity in order to live."

Scientific communications at the meeting fell into three groups: morphological and genetic evolution of lemurs, field studies in 'undisturbed' habitat, and papers which took up Andrianampianina's theme of conservation with its social corollaries.

Morphologists seemed to concur, for once, that the Cheirogaleinae, or primitive mouse and dwarf lemurs, form a very distinct group from the Lemurinae or true lemurs like the familiar ringtail. The same picture emerged from multivariate analysis of limb bones (C. Oxnard, J. McArdle, R. German, Chicago; F. K. Jouffroy, Museum Nationale d'Histoire Naturelle, Paris), from the pelvic girdle (C. Berge, MNHN, Paris), the skull (N. Petit-Maire, CNRS, Marseille), and fingerprint analysis (B. Rakotosamamina, Ministry of Higher Education and Scientific Research, Madagascar). This could mean that the Cheirogaleinae will eventually be elevated to family status, which would give Madagascar four primate families of its own, with the Lemuridae, the leaping Indridae, and the Daubentoniidae. (Of course, *Daubentonia*, the preposterous aye-aye, may be extinct by then.) Randriamanantenina (University of Antananarivo) presented a new analysis of the subfossil genera of giant lemurs which disappeared about 1,000 years ago with the coming of man. She showed that extinct lemurs were robust in allometric proportion to their size, which suggests that there were no agile brachiators among them. Comparing

limb and vertebral indices confirms Walker's view that *Megaladapis* was a koala-like clinger, but *Paleopropithecus* may have hung upside down like a sloth. H. Hemmer (Mainz) discussed brain size in *Lemur* and W. Padushka (Vienna) the evolution of primate scent marking from ancestors like the Malagasy insectivores.

Chromosome and serum transferrin studies indicate that both *Lemur* and *Lepilemur* are in the course of active speciation (J. Buettner-Janusch, New York University; Y. Rumpler, Strasbourg; J. Andrianivo, Hôpital Gerard et Robic, Madagascar). Each is highly polymorphic in chromosome number, with some genetically isolated species such as *Lemur macaco* or *Lepilemur septentrionalis* which are no more different in morphology than the many races or local variants of interbreeding forms. Buettner-Janusch suggests that much of the molecular and chromosomal variation has arisen in the past thousand years, since the Malagasy central plateau was cleared, leaving a ring of semi-isolated lemur populations round the coast. Coulanges (Institut Pasteur, Madagascar) reviewed the literature on lemur disease. Their physiology differs from other primates: they are apparently immune to polio and to falciparum malaria. It is not known what role lemurs have in the transmission of other malarias, or whether the fact that there is no yellow fever on Madagascar is due to lemurs' immunity, to the ecology of the local *Aedes* mosquito species, or to luck.

Field studies dealt with comparative ecology and behaviour of nocturnal lemurs in a highly seasonal western forest (J.-J. Petter, E. Pages, MNHN, Brunoy), with separation of lemur, bird and insect calls by time or pitch such that each species has its own 'acoustic niche' (L. McGeorge, Duke University, North Carolina), and with foraging strategies in *Indri* (J. Pollock, University of Cambridge). Pollock's *indri* even draped themselves on a branch and sang their air-raid siren territorial wails in front of forty conference members, which should infuriate all naturalists who have spent days hunting *indri* in vain. The field work for all those studies, however, was done before the Malagasy Republic suspended research visas for foreigners in 1974. Recently M. Razarihelisoa, R. Albignac, and their students at Antananarivo University have embarked on a study of *Avahi*, the woolly lemur. *Avahi* is nocturnal, lives in pairs in well-defined territories, and eats only leaves. To judge by the conference field trip its movements are as predictable as the

*International Colloquium on Malagasy lemurs, held at Antananarivo, Democratic Republic of Madagascar, on 4-14 September under the sponsorship of the Malagasy Academy.

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teddy-bear it resembles. The study site is an effectively protected forest, a 6-hectare remnant inside the plantation of a paper mill, which has helicopters for fire-fighting. That tiny *Avahi* population may survive, just as white sifaka and ringtailed lemur populations have remained stable since 1963 in the privately protected 100-hectare reserve at Berenty. Lemurs are relatively small and sedentary mammals, so it may be that the minimum critical size of an effective lemur reserve is modest in terms of each species. Larger scale reserves, are needed, instead, to save a representative sample of all the forest types, from rainforest to the thorn-studded, arid, *Euphorbia* forest of the south.

As for direct conservation action, Antananarivo University has just founded a field station with a reserve fenced and guarded on the Berenty model. This is a cooperative project with Yale and Washington (St. Louis) Universities, financed through the World Wildlife Fund and set up by Guy Ramanantsoa (Antananarivo), the conference organiser. After considerable difficulty in finding a local commune which would permit a reserve on its land, he at last chose a stretch of gallery forest in the south, where the local commune welcomed university involvement. This 100-hectare field laboratory is particularly important because it has adequate funds. It is, of course, trivial in scale compared to the 470,000 hectares of designated wilderness reserve which the government has no funds to protect. When Bruno Ratovomboahangy, one of Ramanantsoa's students, described the plight of the rare black sifaka, a race clearly doomed to extinction because its last shred of forest is being cleared, J. Manambolona (Antananarivo), the session's chairman, pointed round the room to four government officials and declared, "This is exactly why we are met, and this is what you must cure!"

Rabe Rafael, Director of Water, Forest and Soil Conservation, presented a detailed proposal for protection of the 580,000 ha of national parks and reserves. He asks for funds amounting to £1.2 million. Less than half that sum would be for the strict forestry needs of personnel and equipment. The other half would go toward formulation of land-use plans and to developing agricultural alternatives for people living around each reserve.

There is now increasing international awareness that the social and economic benefits of conservation are inextricably involved with any park plan. If UNEP, the IUCN, and other international bodies aid ecologically rational development in Madagascar, they could help the Malagasy Republic to save a unique portion of the world's scientific

heritage. If not, the Malagasy will conclude that rich countries only find money to fly their own scientists to conferences. Western knowledge will seem as partial as the expertise of "the man who wears a loincloth that is falling to pieces—he knows exactly how to put it on and how to take it off."

And if nothing is done, the peasants who now roll back the forest will have the same problems in 10 years' time that they do today: eroding watersheds, sterile soil, dwindling firewood supplies. The only answer then will be planted pines or eucalyptus. The option of saving the Malagasy forest communities and hundreds or even thousands of Malagasy species will be gone. □

Halophilic ribosomes

from Richard Brimacombe

COMPARISON of ribosomes from different organisms provides a promising approach to the problem of understanding ribosome structure and function. Several laboratories are engaged on amino acid and nucleic acid sequencing of ribosomal components and are gradually piecing together the information to make such comparisons. One example is recent work on the ribosomal proteins of the extreme halophilic bacterium *Halobacterium cutirubrum* which has come up with some intriguing results. Matheson *et al.* (in *Halophilic Microorganisms* (ed. Caplan) Elsevier/North Holland Biomedical Press (in the press)) have compared the amino acid sequences of the acidic ribosomal proteins—the A proteins—from a variety of halophiles ranging from moderate to extreme. These A proteins are those that seem to correspond with proteins L7/LI2 in *Escherichia coli*.

The ribosome of *H. cutirubrum* has been of interest for some years. Whereas the RNA and protein components are similar in size and number to those of *E. coli*, the proteins are very much more acidic (Bayley & Kushner *J. molec. Biol.* **9**, 654; 1964). It follows that two-dimensional gel electrophoresis gives little clue to which *H. cutirubrum* proteins correspond to which *E. coli* proteins, and the two organisms are sufficiently unrelated that comparison of N-terminal amino acid sequences is also not very helpful. So Matheson *et al.* have restricted their comparative study to the proteins apparently equivalent to *E. coli* L7/LI2 (L20 in *H. cutirubrum*), and to those proteins which bind to

5S RNA. *E. coli* proteins L7 and L12 differ only in the presence of an N-terminal acetyl group on L7, they are distinguished by being the most acidic proteins in the 70S ribosome, and the only proteins present in multiple copies (three to four per 70S). These proteins are functionally important, notably in GTP-dependent processes, and are interchangeable between ribosomes from unrelated organisms such as *E. coli* and yeast (for review see Möller in *'Ribosomes'*, Cold Spring Harbor Press, 711, 1974).

The complete amino acid sequence of *E. coli* L12 (EL12) has been known for some time (Terhorst *et al. Eur. J. Biochem.* **34**, 138; 1973), and Matheson *et al.* find that, whereas the amino acid sequences of the equivalent proteins from moderate halophiles are very similar to that of EL12, the sequence of L20 from *H. cutirubrum* (HL20) (which is now complete except for an unordered highly acidic region between residues 78 and 106) has some very interesting similarities and differences. A simple comparison of the amino acid compositions shows that in HL20 the lysine content has dropped drastically from 13 residues to 1, and there is a corresponding increase of 7 to 22 in aspartic acid residues. 60% of the protein is in fact composed of aspartic acid, glutamic acid and alanine. Further, methyl lysine (present in EL12) is absent, and the valine content is reduced, whereas at the same time two tyrosine residues (absent in EL12) make an appearance. Nevertheless, despite these very large differences, there are still distinct regions of homology between the *E. coli* L12 and *H. cutirubrum* L20 sequences.

The first of these is a region of about 18 amino acids near the N-terminus of HL20 (positions 12–30), which shows a high degree of similarity to EL12, not in the N-terminal region of the latter, but in the middle of the sequence (positions 46–62). Another characteristic region, which is very rich in alanine, is observed in the *H. cutirubrum* protein (positions 57–67, with nine alanines). This region is C-terminal with respect to the homologous region just mentioned, whereas the corresponding alanine-rich region in EL12 (positions 34–42, with eight alanines) is on the N-terminal side. Thus the two homologous regions are inverted, and the whole N-terminal region of the *E. coli* protein seems to have completely vanished from the *H. cutirubrum* protein. In addition the basic area of EL12 (positions 57–84) has disappeared in HL20, the latter in turn showing a highly acidic C-terminal region (positions 70–112) which has no equivalent in EL12.

The sequence of *H. cutirubrum* L20

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shows less homology with the moderate halophiles than it does with *E. coli*, suggesting that the extreme halophile has not evolved from the moderate halophiles. Indeed a comparison with the N-terminal sequences of the corresponding N-terminal sequences from two eukaryotic organisms *Artemisia salina* and *Saccharomyces cerevisiae* (Amons *et al.* *FEBS Lett.* **81**, 308; 1977) shows a high degree of homology with HL20 in the first 11 residues, including the two tyrosine residues mentioned above. It seems therefore that the extreme halophile has both pro and eukaryotic character, an idea which is supported by the studies on the N-terminal sequences of the 5S RNA-binding proteins reported by Matheson *et al.* as well as by the sequence of the *H. cutirubrum* 5S RNA itself. Matheson *et al.* draw attention to the recent work of Magrum *et al.* (*J. molec. Evol.* **11**, 1; 1978), which places the extreme halophiles in the Archaeobacteria, primitive organisms which are little related to either pro or eukaryotes.

The amino acid sequence data are interesting in themselves, but there is another aspect of the *H. cutirubrum* ribosome which is equally important in

comparative studies, namely the halophilic property itself. *H. cutirubrum* ribosomes require at least 3.4 M KCl and 100 mM magnesium for stability, a condition under which 'normal' (that is *E. coli*) ribosomes would lose a substantial amount of their protein. Conversely, under the low salt conditions used for protein synthesis with *E. coli* ribosomes, the *H. cutirubrum* ribosome loses much of its protein (Bayley *J. molec. Biol.* **15**, 420; 1966). L7/L12-type proteins have a very high α -helix content, and have alanine-rich sequences similar to those in motile proteins (Amons *et al.* *FEBS Lett.* **86**, 282; 1978). In the case of HL20, the instability of the ribosome at low salt concentration can be at least partly related to this factor, since Matheson *et al.* find that HL20, unlike EL12, loses most of its helicity at salt concentrations below 2 M.

Thus, *H. cutirubrum* L20 is a protein which is part eukaryotic, part prokaryotic; the prokaryotic region of the amino acid sequence is back-to-front, and the protein has to function in a ribosome whose salt requirements are upside-down. The system offers fascinating possibilities for comparative studies of structure and function. □

Plant cells without walls?

from Jeremy Burgess

ISOLATED plant protoplasts are emerging slowly from their former state as an interesting curiosity and a subject for speculation to a new level of usefulness as a research tool, with their main application in virology and cell wall studies. Protoplasts are particularly valuable to the plant virologist as synchronous infection of a high percentage of a population can be easily achieved. This allows the study of the sequence of events as an infection develops in a way which is all but impossible using whole plants. For cell wall studies, protoplasts offer a rare chance of access to a naked plant cell plasma membrane which will, in some species at least, produce a functioning wall structure after only hours or days of culture.

One assumption underlies all this work on protoplasts, and it is now being brought into question. This is that a protoplast behaves as a plant cell without a wall. Mechanically this definition is certainly correct, but is it accurate in a biological sense? If it is not, and results obtained with protoplasts cannot be applied to whole plants, then the usefulness of the

protoplast as a model system is undermined.

The problem arises out of the way in which protoplasts are prepared. In brief, a tissue such as a leaf is first immersed in a concentrated solution of a sugar to which the plasma membrane is impermeable. This causes the protoplast to shrink away from the wall which bounds it, as water is withdrawn by osmosis. Once plasmolysis has occurred, the wall is enzymatically digested away.

The protoplasts are released into suspension as individual units stabilised against bursting by the sugar solute. So during its preparation the protoplast has been partially dehydrated; the connections between adjacent cells in the tissue, and between each cell plasma membrane and its surrounding wall have been severed and the cell wall has been removed. Naturally it was the third of these processes which concerned early workers with protoplasts. Now it seems that not enough attention has been paid to the effects of the other two.

Several recent papers have cast doubts on the assumption that freshly isolated protoplasts behave simply as plant cells without walls. Ruesink (*Protoplastes et fusion de Cellules*

Somatiques Vegetales, 41, CNRS, 1973) has shown that leucine uptake by plasmolysed protoplasts is drastically inhibited compared with intact cells. Racusen, Kinnersley and Galston (*Science* **198**, 405; 1977) examined electrical potentials in normal and plasmolysed tobacco leaves as well as protoplasts. They were able to show that plasmolysis results in the normal negative potential within the cell being converted into a small positive one. If the protoplasts were cultured for 10-14 days, those which reformed a cell wall also regained the negative potential. By contrast, protoplasts of maize which failed to produce a functional cell wall in culture also failed to regain the normal negative potential (Kinnersley, Racusen & Galston *Planta* **139**, 155; 1978). Changes in levels of protein synthesis and photosynthetic rates also occur in plasmolysed cells. These effects of plasmolysis on the general vitality of protoplasts are not immediately reversed. Premecz *et al.* (*Plant Sci. Lett.* **9**, 195; 1977) used RNase levels as a measure of response to stress and found that the concentration of plasmolysing solution used affected the time taken for the recovery of normal physiology. It seems clear that the initial plasmolysis and the subsequent culture in a concentrated sugar solution both present the protoplast with a stress which alters much of its biochemical behaviour compared with the normal plant cell. Thus freshly isolated protoplasts should not be regarded as plant cells without walls, but as a stressed system requiring a recovery period. For example, we have shown the need for such recovery period in the case of cell wall growth on tobacco leaf protoplasts (Burgess *et al.* *Planta* **139**, 85; 1978). Although on first isolation an 8-hour lag precedes visible wall formation, subsequent removal of this wall without imposing additional osmotic stress on the protoplasts allows a new wall to begin to form within 12 minutes.

There is no doubt that in the long term (a period measured in days rather than hours), protoplasts from many sources can completely overcome the effects of osmotic stress. Whole plants have been grown from single protoplasts in a handful of species, and many others will regenerate walls and divide, even if the cells subsequently fail to differentiate into organised tissue. However, most of the current interest in protoplasts is centred on the initial minutes or hours following isolation. This is the period when they are amenable to virus infection, when fusion frequencies are at their highest, and when the first cell wall components begin to be secreted across the membrane. That this is a period of high

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stress for the protoplast should not be overlooked. One area of growing interest is the isolation of the plasma membrane to examine its properties with regard to the synthesis of cell wall components, and also its interaction with virus particles. For results from this kind of study to have meaning in the context of the intact plant cell, allowance must be made for the effects of osmotic stress and dehydration. At present there seem to be two ways of minimising these difficulties. The first is to use a second enzyme treatment on protoplasts which have recovered for a few hours in culture. This approach, however, assumes that the protoplasts will recover. The second way might be to ensure that the cell is not so greatly stressed initially. Colman and Mawson (*Z. Pflanzenphysiol.* **86**, 331; 1977) have found that gradual plasmolysis of leaf tissue is necessary for the isolation of photosynthetically active cells. Such a refinement in technique applied to protoplast isolation might well increase the range of species which will respond to culture. Any improvement in the physiology of freshly isolated protoplasts is to be warmly welcomed. □

Nucleon-nucleon interactions

from a Correspondent

THE study of the forces between free nucleons, at energies not much greater than their rest masses, falls between the present interests of nuclear structure and of elementary particle physics. Administratively this is awkward and in Britain nucleon physics is neither popular nor lavishly funded. However, since the 'meson factories' in several other countries began operating a few years ago there has been a resurgence in studies of this fundamental interaction, which is now sufficiently well understood that we are on the verge of an accurate and coherent account of the properties of light nuclei, and of the simplest processes of meson production. These facts, which had been hinted at a year ago at the Vancouver Conference on the Nucleon-Nucleon Interaction, emerged clearly at a recent meeting in Graz*.

At all energies up to about 400 MeV proton-proton elastic scattering is accounted for by the exchange of virtual mesons, together with a set of unambiguous and well-determined phase shifts in the S and P partial waves.

The fine details of this phenomenology are due to measurements at small scattering angles by R. Hess's group from the University of Geneva, who have measured a variety of spin-dependent variables at SIN, Switzerland; and from similar measurements, over a wider angular range, performed at TRIUMF, Vancouver, by the BASQUE group (D. V. Bugg *et al.*; Queen Mary College, London; University of Surrey; Universities of British Columbia and Victoria). So well are these data described by boson exchange potentials (as developed, for example, by R. Vinh Mau's group at IPN, Orsay) that, in the opinion of G. H. Thomas (Argonne National Laboratory) no further measurements of this type are required.

Until very recently the corresponding neutron-proton data have been of poor quality, the experiments being more difficult and the available neutron beams less intense. At Graz, new measurements of triple scattering parameters were reported by the BASQUE group (D. V. Bugg *et al.*; and of the differential cross section for charge exchange, by both E. Rössle's University of Freiburg group, working at SIN, and a LASL/Texas A & M/University of Texas team at LAMPF, Los Alamos (B. E. Bonner *et al.*). The remarkable agreement between the two sets of cross sections disposes of the long-outstanding uncertainty about this quantity. The quality of these data, and of the BASQUE measurements, is now as good as the proton-proton data, and illustrate the capabilities of the accelerators at TRIUMF, SIN and LAMPF.

The BASQUE measurements have been incorporated in a number of partial wave analyses, and as R. A. Bryan (Texas A & M) showed, not only are the isosinglet phases now unique below 400 MeV, but the theoretical interpretation has altered significantly in the past year to give excellent agreement with the new data. This is seen, not only in the work of Vinh Mau's group, presented at Graz, but also in some recently-published work by the University of Florida group (Veda & Green *Phys. Rev.* **C18** 337; 1978). Likewise, near 50 MeV, a careful set of measurements by F. P. Brady *et al.* (University of California, Davis) has clearly exposed the incorrectness of earlier data, which had previously been an embarrassment to the theoreticians.

With such excellent understanding of elastic nucleon-nucleon scattering, the way is now open to incorporate these theoretical models into the potentials used in nuclear structure calculations. This was made clear in a lucid review by K. Hollinde (Bonn

University). Incidentally, the perennial fear that off-mass-shell effects may dominate such many-body systems seems groundless; the considerable effort devoted to nucleon-nucleon bremsstrahlung has failed to turn up any evidence for gross departures from elastic amplitudes. Though off-mass-shell effects exist (they are needed, for example, to account for the cross-section for the capture of thermal neutrons on hydrogen), they are clearly weak at the energies considered here.

At higher energies, uncertainties multiply. Are there resonances in one or more partial waves, for example the 3F_3 and 1D_2 , as N. Hoshizaki (Kyoto University) has suggested in a recent preprint? The phase-shift analyses are ambiguous above the inelastic threshold. Using a coupled channel approach, E. Lomon (Massachusetts Institute of Technology) was able to relate the BASQUE data below 500 MeV to possible resonances above this energy. Di-baryon resonances can appear in quark models, and this was discussed in several contributions, such as those of J. J. de Swart (Nijmegen) and C. de Tar (MIT). What is needed are better experimental data on spin-dependent amplitudes near 1 GeV, and so far only the ZGS at Argonne has provided this. The untimely shutdown of the ZGS next year will leave Saturne II, the revamped accelerator at Saclay, alone in this field, and the nucleon-nucleon programme there, under F. Lehar, should be of great interest.

The key to our understanding lies in the inelastic processes. J. A. Niskanen (University of Helsinki) has developed a model of pion production in p-p scattering which goes well beyond the simple Mandelstam model of 25 years' standing, by incorporating explicit isobar components into the nucleon configuration. G. Jones (University of British Columbia) reported on measurements from TRIUMF which were in strikingly poor agreement with Niskanen's model. But Mandelstam's model itself is inherently unsatisfactory; it predicts that pion production in n-p collisions is due only to the isovector part of the amplitude. E. Rössle's group presented data taken at SIN which seem to contradict this, though the experiments, and their interpretation, are difficult. Their results, and those of Jones, also show compelling evidence for d-wave pion production, even very close to threshold.

If the scattering states of two nucleons are so well understood, why is the admixture of D-state in the bound system, the deuteron, so poorly known? The answer is that the D-state arises from a tensor coupling, which is due to the exchange of rather heavy

* The 8th International Conference on Few Body Systems and Nuclear Forces was held on 24-31, August in Graz, Austria.

mesons which evoke short range forces; and the interaction at small distances ($\lesssim 0.8$ fm) is not well understood. M. Fiedeldey (Pretoria) showed that several ingenious suggestions for enhancing the experimental sensitivity to the D-state were impracticable, though D. Roger *et al.* (CEN, Saclay) showed that the quasi-free electron scattering experiments now command the accuracy needed for the task. It remains a tantalising problem.

Neutron-deuteron backward scatter-

ing was studied by Bonner's and Rössle's groups at Los Alamos and SIN. Their data again agreed well, and the interest here was in a departure of the cross section from a smooth exponential near 180° . This unexpected behaviour occurs between 380 and 650 MeV. No doubt by the next Conference in this series, scheduled for 1980 at Eugene, Oregon, explanations will have been proposed; new puzzles will also have arisen in this exciting, and exacting, field. □

Rats, pigs and men in New Guinea

from Robert M. May

MAN'S impact on the New Guinea highlands dates back five or six thousand years, when widespread development of a shifting slash and burn agriculture led to substantial forest clearance, and, at the same time, the domestic pig was introduced (Bulmer *J. Soc. Océan.* 31, 7; 1975). Over the subsequent millennia, patterns of sedentary settlement gradually emerged. Major disturbance of habitats is, however, probably relatively recent, stemming from the introduction of the sweet potato (*Ipomoea batatas*) as a major food crop some 300 to 400 years ago; this innovation led to increases in population density and to the development of intensive pig-keeping at higher altitudes.

The most conspicuous change wrought by human activities in the New Guinea highlands is the replacement of forest by grassland, maintained by intermittent burning. These increasing areas of man-made grassland exist in a flux of various successional stages, as old gardens or village areas are abandoned and new ones created. The associated long-term changes in the distribution of animal species are shown by recent archaeological studies. One revealing study is from a site at Aibura, in the Eastern Highland Division, where it can be seen that over the past 1,000 years the forest-dwelling phalangerines (for example, *Phalanger* or cuscus species) have declined while the grassland pademelon (*Thylogale*, a relative of the rock-wallaby) has concomitantly increased (White, *Ol Tumbuna: Terra Australis* No. 2, Australian National University, 1972).

A particularly detailed account of the present-day distribution patterns of seven species of terrestrial rats in relation to the changing habitat in the New Guinea highlands has recently been given by Dwyer (*Aust. J. Ecol.* 3,

213; 1978). The seven species all belong to the Old World family of murid rodents, four from the genus *Rattus* and three from *Melomys*, as tabulated below. The 'productivity index' is a measure of the intrinsic capacity for population growth of the species, which Dwyer computes by multiplying the mean litter size by the average proportion of females (above minimum adult weight) that are in breeding condition. By thus using the proportion of adults that are breeding, Dwyer takes some account both of the number of litters per year and of the age structure; the quantity is, moreover, weighted to allow for differences between wet and dry seasons.

Species	Productivity index
<i>R. exulans</i>	2.10
<i>R. ruber</i>	1.74
<i>M. rufescens</i>	1.18
<i>R. verecundus</i>	0.94
<i>M. levipes</i>	0.60
<i>R. niobe</i>	0.42
<i>M. rubex</i>	0.35

As a very crude generalisation, we might expect the successful species in the relatively more disturbed and unpredictable grassland habitat to tend towards the 'r-strategy' of 'high reproductive rates, short life spans, large population size, . . . and broad, often cosmopolitan distribution' (Grassle & Saunders *Deep Sea Res.* 20, 643; 1973), while the successful species in the relatively undisturbed and predictable forest habitat may tend towards the opposite 'K-strategy' (for more full exposition of these notions, see *Nature* 257, 737; 1975; 267, 394; 1977). Indeed Felming (*Ecology* 55, 555; 1974) has interpreted the life history strategies of two species of heteromyid rodents in Costa Rican rainforests in this light: *Liomys salvini* in the seasonal highland

forest as an r-strategist and *Heteromys desmarestianus* in the season-free lowland forest as a K-strategist. Similarly Freeland (thesis, Queensland University, 1972) has suggested that two murids in a subtropical Eastern Australian rainforest coexist by virtue of *Rattus fuscipes* exploiting an r-strategy keyed to environmental features that are unpredictable while *Melomys cervinipes* is adapted to relatively predictable features. If these simple ideas are to apply to Dwyer's more complicated seven-species system, we expect the habitats to manifest a decreasing degree of disturbance as we run down the species list in the table above; that is, we expect the 'productivity index' to be significantly larger in more disturbed habitats.

Dwyer shows this is indeed so. His extensive trapping studies in the Mt Erimbari-Mt Homo region at the western boundary of the Eastern Highland Division of Papua New Guinea show that the short grass communities (which are created and sustained by man) support only *R. exulans*. Notably, this is the only species on the list that also lives in houses. *Miscanthus* communities represent a later successional stage that is still short grass, and they are also largely under human control; here are found *R. ruber* and *M. rufescens*. *R. verecundus* and *M. levipes* are found in the extensively disturbed oak-dominated forests of Mt Homo, and *R. verecundus* and *M. rubex* occur together in the lower and more disturbed forests of Mt Erimbari; *R. verecundus* is more tolerant of areas regularly disturbed by pigs. High on Mt Erimbari, *M. rubex* and *R. niobe* are found together where disturbance by humans and pigs is least. The few trappings of *R. niobe* below 2,400 m were from well-protected sites. *R. niobe*, in particular, seems to need dense cover of the kind that is incompatible with piggly attention; the existence of this species elsewhere in high (and undisturbed) grassland and in forests at lower altitudes shows that neither altitude nor vegetation alone confine it within the narrow bounds observed in Dwyer's study area. Other aspects of the distributional ecology also fit the scheme: the density of *R. exulans* in grassland was 20 per ha, and of *R. niobe* and *M. rubex* on an equal grid in mixed forest were 8.0 and 4.6 respectively; the mean monthly survival probability for *R. exulans* in grassland is around 0.77, and for *R. niobe* and *M. rubex* in mixed forest it is 0.90 and 0.92 respectively. In short, these gross patterns of distribution correlate remarkably well with the 'productivity index' tabulated above. Insofar as there are discrepancies, they are that for a ranking based on habitat, *R. verecundus* might rank below *M.*

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levipes, and the reclusive *R. niobe* below *M. rubex*; these departures are easily rationalised by appeal to differences between genera (for example, each *Melomys* is larger than its *Rattus* partner).

Additional support for the idea that the distribution patterns are governed by the amount of disturbance by man and pigs comes from the archaeological record. White's (*op. cit.*) excavations have shown a marked increase in the representation of small rodents in the upper horizons of his site. Tooth measurements suggest that the three species present are *R. niobe*, *R. exulans* and *R. ruber*; of these, the first is found only in lower levels, the second only in the top level, while the third increases over time in step with increasing signs of human activity.

Dwyer concludes "that the local diversity of small terrestrial rodents in rain forests of densely populated areas of the New Guinea highlands was higher in the past than it is today and that reduction has been a consequence of habitat disturbance by men and pigs producing a smaller, less continuous, area of rainforest and simplification of ground cover. The pattern of local species distributions is now dominated by gross disturbance effects with the more opportunistic of rainforest species found in the most disturbed rainforests. Some species may have dropped out of local faunas." He also suggests that some aspects of the distribution patterns of the montane avifauna of New Guinea (as extensively studied by Diamond and others) may be associated with recent disturbances rather than with long term 'island equilibrium' effects. In common with many other workers, Dwyer sees disturbance as important in promoting and maintaining species diversity, essentially because it enlarges the number of different roles in the evolutionary play. But severe disturbance, of a kind outside the evolutionary experience of the ecosystem, will make for less diversity.

The asthenosphere as point defect zone

from Peter J. Smith

At depths in the Earth of somewhere between 75 km and 250 km there is a layer perhaps some 75-150 km thick in which seismic velocity is slightly lower, and seismic attenuation is rather higher, than in the regions immediately above and below. Any uncertainty implicit in this statement arises because the depth and thickness of the layer, which may in any case be variable,

have not been determined everywhere and because, even where experiments have been carried out, the boundaries of the layer are often diffuse. Nevertheless, there seems little doubt that this 'low velocity layer' exists in some form beneath most of the Earth's surface. Moreover, the layer is now usually equated with the asthenosphere, the semi-mobile zone which allows the rigid tectonic plates of the overlying lithosphere to move.

But accepting the identity of the asthenosphere and the low velocity layer, what is the physical cause of the mobility, the lower velocity and the higher attenuation? The majority answer is partial melting of peridotite. Suggestions about the nature of the cavities in which the melt fraction supposedly resides have included spherical pores, coin-shaped cracks and tubular channels; and estimates of the amount of partial melting accordingly required to account for the seismic observations have ranged from about 6% to 0.1%. Whatever the cavity shape, the degree of partial melting required is apparently low; and so if the low velocity zone were purely a static phenomenon there would be little to choose between models.

The reality is more complicated, however, for the molten material can evidently flow, which means that in at least some parts of the asthenosphere the melt cavities must be interconnected. And that seems to pose quite a problem. For according to Shaw (*Geophys. Res. Lett.* **5**, 629; 1978), Walker *et al.* (in press) have investigated the stability under gravity of a melt-solid system with interconnected porosity and have thus shown that for tubular channels, instability will arise if the melt fraction exceeds 0.1% (which compares with the roughly 1% required to explain the seismic observations). Above 0.1% the melt segregates and rises from the solid in less than 10^7 yr. Walker and his colleagues therefore conclude that the low velocity layer is either a temporary phenomenon which just happens to be well developed at the moment or a "dynamic system with a melt fraction above their calculated limit of long term stability through which melt must flow continually at a substantial rate, in fact, at a rate which is probably unrealistically high".

Of course, many assumptions are involved, any one or more of which may turn out to invalidate the Walker model as a reasonable semblance of reality. Nevertheless, at the very least the results are such as to suggest that partial melting should not be accepted

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too readily, as, indeed, some workers have long realised. Guegnen and Mercier (*Phys. Earth Planet. Interiors* **7**, 39; 1973), for example, listed several other possibilities and concluded that the seismic properties of the low velocity layer could in theory be as well explained in terms of viscous grain boundary relaxation or dislocation-impurity interactions.

Unfortunately, there is little experimental data to support either of these particular solid state processes as a cause of the asthenosphere; but Shaw has now added one phenomenon for which there is some empirical (and theoretical) support: namely, the presence of point defects. Shaw himself (*J. Phys. Chem. Solids* **35**, 911; 1974) has shown that in the B1 phase of silver iodide, variation in the concentration of lattice point defects leads to a very rapid decrease in sound velocity with increasing temperature and an equally rapid increase with increasing pressure. The magnitudes of defect-produced velocity changes in materials likely to be important in the Earth (such as silicates and oxides) are far less well known and may in any case be different for different types of defect (Frenkel defects, Schottky defects for example). But Shaw has little difficulty in showing that, taking into account the probable relative temperature and pressure effects in materials and the variation of temperature and pressure in the Earth, a seismic velocity anomaly in the Earth would be expected in the depth range 50-250 km—precisely where it is observed.

Considering the uncertainties involved in Shaw's theoretical development, this agreement is amazing. And it is all the more encouraging in that defects are known to facilitate material transport, thus enhancing flow and leading to a decrease in apparent viscosity. If Shaw is right in his thinking, the presence of point defects offers just as reasonable an explanation of the low velocity layer-asthenosphere as does partial melting, although to prove the point there will have to be far more experiments on probable Earth materials at the appropriate temperatures and pressures. □

DNA polymerase and mutation

from Bryn Bridges

THE underlying importance of damage to DNA and its mutagenic consequences in the production of cancer and certain heritable diseases becomes steadily more apparent, and it may well extend to such superficially unrelated

diseases as atherosclerosis and certain neurological conditions. The steps by which damaged DNA generates permanently altered nucleotide sequences are, however, obscure in mammalian cells. The question is probably closest to resolution in bacteria where there seems to be an intriguing interaction between DNA damage and the fidelity of DNA polymerases.

Ultraviolet light is commonly used as an experimental DNA damaging agent and initiates mutagenesis through constitutive or inducible pathways depending on the circumstances. The existence of this inducible pathway, proposed and termed 'SOS' repair by Miroslav Radman, is best illustrated by the fact that UV-irradiation of bacteriophages λ or ϕ X174 leads to the production of mutations only if the phages are plated on bacteria that have themselves been UV irradiated or given some other 'inducing' treatment.

A mechanism for the action of DNA polymerases in ultraviolet mutagenesis has now been proposed by Radman's group (Villani *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 3037; 1978) who have shown that photoproducts produced in the DNA of ϕ X174 by ultraviolet constitute a block to DNA replication by cell-free extracts of *E. coli* operating either through DNA polymerase I or III. (Recent studies have pointed towards the role of DNA polymerase III in bacteria as the mutagenic enzyme, particularly in the inducible pathway (see Bridges & Mottershead *Molec. gen. Genet.* **162**, 35; 1978)). In experiments with purified DNA polymerase I (large fragment) they further showed that DNA synthesis on an irradiated ϕ X174 primed template was accompanied by a large turnover of nucleoside triphosphates into monophosphates, in contrast to synthesis on an unirradiated template.

DNA polymerase I large fragment lacks 5' to 3' exonuclease activity but retains both polymerising and 3' to 5' exonuclease activities. The latter is believed to act as a 'proof-reader', excising newly incorporated terminal bases which are not paired correctly with the template base. Villani *et al.* suggest that ultraviolet photoproducts in the template strand do not prevent incorporation by the polymerase function but that the bases incorporated opposite photoproducts are registered as mismatched and are immediately excised by the proof-reading exonuclease. The net result is that nucleoside triphosphates are converted to monophosphates with no DNA chain elongation, a condition termed polymerase 'idling'. They further suggest that newly-incorporated mismatched

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bases could persist and be seen as mutations if SOS induction involved the inhibition of the proof-reading function so that chain elongation could proceed beyond the photoproduct and its potentially mismatched partner. In support of this they give evidence that AMV reverse transcriptase, an error-prone DNA polymerase devoid of any exonuclease activity, is both highly prone to insert wrong bases and replicates UV-irradiated ϕ X174 DNA far above the level of *E. coli* DNA polymerase I.

The findings of Villani *et al.* suggest a possible signalling mechanism for triggering the SOS inducible pathway. SOS mutagenesis in *E. coli* is dependent on the *recA*⁺ gene and it has been thought to be similar in its operation to other *recA*⁺-dependent phenomena such as prophage induction. The *recA*⁺ protein is produced in substantial amounts after ultraviolet irradiation and seems to act in prophage induction by carrying out proteolytic cleavage of the λ repressor. Villani *et al.* suggest that the nucleoside monophosphates produced by idling polymerases may well be the initial inducing signal leading to enhanced production of *recA*⁺ protein and thus to the cleavage of λ and other SOS repressors.

Another, simpler possibility is apparent, however, if we consider the recent work of Byrnes *et al. (Biochemistry* **16**, 3740; 1977) who have shown that nucleoside 5'-monophosphates inhibit specifically the 3' to 5' exonuclease but not the polymerase function of DNA polymerase I, and of Que, Downey and So (*Biochemistry* **17**, 1603; 1978) who have further produced evidence that the nucleoside 5'-monophosphates bind at the primer terminus site for the 3' to 5' exonuclease activity. On the basis of their observations one might argue that SOS mutagenesis induction might be more simply explained as follows. Arrival of a photoproduct at the replication fork causes polymerase III idling and accumulation of nucleoside monophosphates. These in turn directly inhibit the 3' to 5' exonuclease function ('induction') and allow chain elongation to continue with any mismatched bases (both opposite the photoproduct and elsewhere) persisting as mutations. Return to high fidelity replication would occur after normalisation of the nucleoside monophosphate pool. The elementary hypothesis of direct control by nucleoside monophosphate levels suggested here does not, however, have any obvious requirement for repressor cleavage by the *recA*⁺ protein. Although it may seem heretical, perhaps the possibility should be considered that the *recA*⁺ protein does not act proteolytically in ultraviolet mutagenesis but rather interacts directly at the DNA level to

'stabilise' the photoproduct-primer terminus configuration so as to allow polymerase idling. Consequent elevation of nucleoside monophosphate levels would then result in direct inhibition of the 3' to 5' exonuclease function and this in turn would lead to mutagenesis and survival. Certainly a constitutive level of the *recA*⁺ protein exists and the protein is known to bind to single-stranded DNA. Furthermore the γ -irradiation resistance conferred by the *recA*⁺ gene seems to be independent of protein synthesis and does not involve inducibility. Could this also be true for ultraviolet mutagenesis? Such an explanation, while based on slender experimental evidence, would seem to merit consideration, if only because of its simplicity. One would still have to find a role on either hypothesis for other genes (*lexA*, *lexC*, *umuC*, and *recB uvrD*, for example) which affect ultraviolet mutagenesis.

Whether or not the postulated inhibition of the proof-reading exonuclease function by nucleoside monophosphates is direct or indirect will doubtless become clear in the not too distant future. The concept of polymerase idling proposed by Radman's group is, however, very plausible and should stimulate the necessary experiments, particularly those designed to reveal whether it occurs *in vivo* as well as with the purified enzyme fragment. Also to be resolved is the question of whether DNA polymerase III behaves *in vivo* the way the polymerase I fragment behaves *in vitro*, and the question of constitutive mutagenic repair should this, as seems likely, also be mediated by polymerase III. Does this also involve inhibition of the proof-reading function and if so, how?



A hundred years ago

CAPTAIN PATTERSON, Superintendent of the U.S. Coast Survey, has lately initiated a very important undertaking in connection with the work of the Survey, namely, in determining the extent and position of the oyster beds of the Chesapeake Bay, primarily with reference to the formation of oyster reefs, and their interference with navigation, but broad enough in its scope to serve as the basis of a critical investigation of the whole subject of the oyster fisheries and oyster culture in the United States. The work is being prosecuted in the Chesapeake Bay by the Coast Survey Steamer *Palinurus*, Mr. H. J. Rice, formerly of Johns Hopkins University, looking more particularly after the natural history features, such as the embryology and development of the oyster, &c. From *Nature* 18, 17 October, 653; 1878.

review article

Steroid-induced meiotic division in *Xenopus laevis* oocytes: surface and calcium

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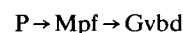
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Progesterone reinitiates meiotic maturation in Xenopus laevis oocytes. Evidence is reported which indicates that the steroid acts at the level of the cell surface and suggests that an induced change of Ca^{2+} distribution triggers in turn a cascade of cytoplasmic events including protein synthesis and germinal vesicle (nucleus) breakdown. These novel features of steroid hormone action in amphibian oocytes are discussed in relation to presently accepted views of the mechanism of action of steroid hormones in somatic cells.

IN TARGET cells, hormone-receptor complexes interact with an effector structure according to two major patterns. The first one, for polypeptide hormones and neuromediators, involves interaction at the plasma membrane level influencing membrane enzyme(s) (for example, adenylate cyclase) and/or cation transfer system(s). In the other case, steroid and thyroid hormone-receptor complexes interact in the cell nucleus with elements of the gene expression machinery as yet poorly defined, and probably modify transcription. We propose here that progesterone action on *Xenopus laevis* oocytes is initiated at the surface level of these germ cells. The effects differ in many important respects from what has been described in steroid hormone target tissues, and they can be mimicked or inhibited by agents which operate at the membrane level and/or induce changes of intracellular Ca^{2+} distribution.

Fully grown *Xenopus laevis* oocytes are arrested at the prophase of the first meiotic division; at this stage 'VI' (ref. 1), they have a diameter of about 1.2 mm, vitellogenesis is completed and the large nucleus (500 μ m) contains replicated DNA. They can be obtained free of follicle cells by appropriate collagenase treatment and can be cultured at room temperature. Progesterone (P) added to the medium induces 'meiotic maturation' *in vitro*²⁻⁴, which proceeds to the metaphase of the second meiotic division. The effects produced by the steroid include the breakdown of the nucleus or germinal vesicle (Gvbd), chromosome condensation, spindle formation and cytokinesis, with the production of an egg ready to be fertilised and the ejection of a small polar body. These events are associated with an increase of proteins related to meiosis, and eventually fertilisation and early embryonic development⁵⁻⁹; protein synthesis seems to be independent of transcriptional control throughout meiosis⁵⁻¹⁰. The eggs obtained *in vitro* are indistinguishable from those produced *in vivo* after gonadotropin stimulation of follicle cells where progesterone is released for direct delivery to the oocyte (without passing through the blood)¹¹; they are able to support cleavage and development after receiving a transplanted blastula nucleus¹².

With progesterone in the medium at 1 μ M concentration, Gvbd occurs *in vitro* after 5-8 h and is easily identified by the white spot observed on the pigmented animal pole of oocytes. During this period, the cytoplasm acquires a 'maturation promoting factor' (Mpf)^{4,13,14}, which can directly induce Gvbd when injected into an untreated oocyte. These results suggest the following sequence:



Mpf is neither progesterone nor a metabolite of progesterone. Indeed, progesterone does not provoke Gvbd when injected directly into the oocyte. It does not act through a nuclear mechanism, and can induce Mpf in enucleated oocytes and in actinomycin D or ethidium bromide treated oocytes. In cells exposed to cycloheximide, the hormone is ineffective; however, Mpf injected into such oocytes produces its normal effect^{12,15}.

We are concerned here with the mechanisms by which progesterone triggers the formation of Mpf, proteins and eventually Gvbd in the incubated oocytes.

Progesterone concentrations required

When oocytes are exposed to different concentrations of progesterone for 8 h, a dose-effect relationship is observed using Gvbd as response (Fig. 1). The half-maximum effective concentration (ED_{50}) is of the order of 10-100 nM. The dose-response curve depends on the time of progesterone exposure: if one observes Gvbd after a much longer time (24 h), lower concentrations of progesterone are found to be active. The 'real' apparent interaction constant of the hormone with the oocyte is difficult to calculate because Gvbd is a late event far removed from progesterone interaction with the oocyte, and as, according to the hormonal state of the female, oocytes are ready to 'mature' more or less rapidly, then each experiment must have its internal control.

It has been reported that the degree of progesterone stimulation of maturation is not dependent on its molar concentration, but rather on the actual quantity of hormone available, and the

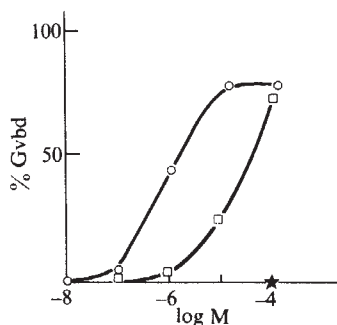


Fig. 1 Progesterone and ACA-poly (EO) activation of meiotic maturation expressed as the % of oocytes undergoing Gvbd. At each experimental point, 100 oocytes were incubated continuously with the inducer (concentrations in incubation medium at zero time), and the % of Gvbd was scored after 16 h exposure. Each point represents the mean of six different experiments on different females. ED_{50} for ACA-poly (EO) ($\square$) and for progesterone ($\circ$) were, respectively, 30 and 2 μ M. 0.1 mM O_2 -poly (EO) ($\star$) did not induce maturation.

incubation medium volume for a given concentration¹⁶. However, when enough time is allowed for observing completion of the maturation, the same efficiency is obtained whatever initial volume is used (Fig. 2). Moreover, incubation of oocytes with a polymer-bound steroid (Fig. 3 and below) does not display the apparent volume dependence of progesterone for short incubation periods (Fig. 2); the explanation for this difference may be that the polymer-bound steroid, in contrast to progesterone, cannot enter the cell and subsequently does not undergo trapping and/or transformation to inactive metabolites or metabolites less active than the parent compound.

Steroid specificity of agonists

Many steroids other than progesterone are very active (Table 1). The hormonal specificity is very different from that observed in progesterone-sensitive somatic cells. (This is based on observations in mammalian and avian progesterone target tissues, as we are not aware of similar studies in amphibians.) Thus, testosterone, deoxycorticosterone (acetate) and cortisol are very active. Certain 5α and 5β -reduced metabolites of progesterone and testosterone are also active, even though they cannot be metabolised back to the parent hormone. Pre-hormones, which may be transformed into active hormones, are also agonists. However, it must be emphasised that not all steroids are active. Oestradiol is not an agonist, and, as seen on Table 1, only some

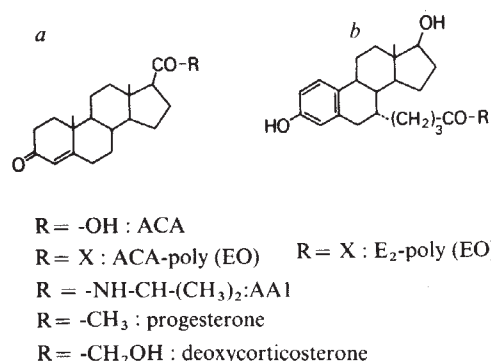


Fig. 3 Proposed structure of the steroid derivatives. $X = -NH-(CH_2)_3-NH-(CH_2-CH_2-O)_n-CH_2-NH-(CH_2)_3-NH-$; ACA = androsta-4-ene-3-one-17- β -carboxylic acid; EO = amino-propyl-polyethylene-oxide. Note that in the case of E_2 -poly(EO), the MW of poly(EO) is 6,000, whereas for ACA-poly(EO), the MW of poly(EO) is 20,000 (see ref. 23).

Despite these difficulties, it has been possible to show that some synthetic steroids which are inactive or weak inducers of meiosis even at very high concentrations, can act as antagonists of progesterone action. Data concerning R-2341, a potent androgen in mammalian tissues, are reported in Fig. 4, and effects of other steroids are shown in Fig. 5. It is also remarkable that we have observed inhibition of progesterone action with oestradiol and other oestrenestroids (Table 1), but not with diethylstilboestrol, a nonsteroidal oestrogen. Together with the fact that cholane and cholestane derivatives are neither active nor antagonistic, the results give evidence for some type of specific binding site for progesterone action in oocytes which differs profoundly from that known in classical progesterone target tissues.

Site of progesterone action

From the lack of Gvbd after progesterone injection into oocytes, it has already been suggested that progesterone may interact with the surface membrane to exert its effects (refs 13, 18), and no specific binding of progesterone has been found in soluble extract from oocytes. However, microinjected cortisol induces maturation, perhaps due to different physicochemical properties¹⁹. The argument based on the nonactivity of injected hormone for designating the membrane as the site of action is not very strong, as injected Ca^{2+} does not give Gvbd, whereas calcium introduced into the cell by iontophoresis is active.

Fig. 2 Time-dependent induction of maturation in the presence of different initial doses of progesterone or polymer-bound steroid, as expressed as Gvbd. 100 oocytes were incubated in 1 (---) or 10 (—) ml of Barth medium in the presence of $\blacktriangle$, 1 μ M progesterone; $\square$, 0.1 mM progesterone; $\bullet$, 0.1 mM polymer-bound steroid (see Fig. 3).

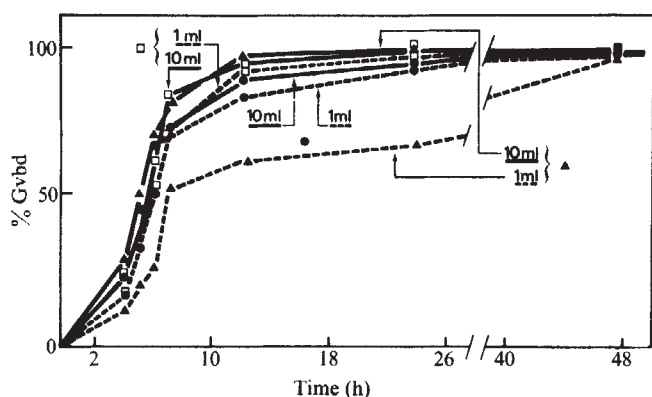


Table 1 Steroids reinitiating meiosis in *Xenopus laevis* oocytes *in vitro*

Agonists	
Progesterone	The most active natural hormone.
17,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione (R-5020)	A synthetic progestagen highly active in mammals.
Deoxycorticosterone (and acetate), cortisol, testosterone	Hormones nonphysiologically related to maturation.
Δ^5 -pregnenolone, Δ^4 -androstenedione, dehydroepiandrosterone	Prehormones, metabolisable to progesterone or testosterone.
5 α -, 5 β -dihydroprogesterone, pregnandiol, 3 α -hydroxy-5 α -pregnane-11,20-dione* 5 α -, 5 β -dihydrotestosterone, 5 α -androstan-3 α ,17 β -diol	Metabolites of progesterone or testosterone, not metabolisable back to hormones.
Cyproterone	Progesterone derivative, anti-androgen in mammals.
19-nortestosterone†(17 β -hydroxy-oestra-4-ene-3-one)	A synthetic androgen.
Non (or weakly) agonists, but antagonists	
16- α -methyl- or ethyl or ethynyl-17 β -hydroxy-oestra-4-ene-3-one, 17 β -hydroxy-oestra-4,9,11-triene-3-one†(R-2341)	Synthetic derivatives of the 19-nortestosterone series.
Oestradiol, 17 α -ethynyl-oestradiol, estradiol-17 β -valerianate, 8-iso-oestradiol	Natural and synthetic oestrogens.
Neither agonists nor antagonists	
13 β -propyl-cortisol, 13 β -ethyl-oestra-4,9-diene-3,17-dione, 17 β -hydroxy-13 β -ethyl-oestra-4,9,11-triene-3-one-17 α -ethynyl (R-2323)	Synthetic derivatives with a 2C or 3C-13 β side-chain (anti-progesterone in mammals).
Diethylstilboestrol	Synthetic nonsteroidal oestrogen.
Cholesterol, cholesta-4-ene-3-one, deoxycholic acid	C27 and C25 compounds.

* Used as an anaesthetic.

† Activities also described in ref. 65.

Melanosomes, however, have been shown to display a selective affinity for active steroids²⁰. This observation is difficult to interpret and cannot be considered to be due to melanin²¹, as oocytes of albino mutants also respond to progesterone.

Our experiments^{22,23} support the hypothesis that the decisive site of progesterone-oocyte interaction is at the surface level. Thus, it has been shown that a progesterone analogue, when linked to a macromolecule, is able to promote maturation without significant entry into the cell. The free steroid (androsta-4-ene-3-one-17 β -carboxylic acid, ACA in Fig. 3) is approximately as active as progesterone on oocytes. When it was linked to a soluble polymer (aminopropylamino-poly(ethylene oxide) poly (EO) of MW 20,000) the complex was found to provoke meiotic maturation with an ED₅₀ of approximately 3 μ M in 16-h incubation experiments (Fig. 1). All effects obtained with progesterone on protein synthesis and Mpf formation were also observed with the steroid-polymer.

Fig. 4 R-2341 inhibition of the progesterone-dependent release of meiosis in the oocytes of *Xenopus laevis*. The data represent the % of Gvbd of 100 oocytes incubated at room temperature in 10 ml Barth medium (pH 7.4). The oocytes are all taken from the same female for each type of experiment (a, b). Maturation was scored after 30 h continuous exposure to progesterone. R-2341 was added to the oocytes 16 h before progesterone. a, Frequency of oocyte maturation induced by 50 nM progesterone as a function of R-2341 concentration. b, Effects of progesterone on the frequency of oocyte maturation in the absence (■) or presence (●) of 2.5 μ M R-2341.

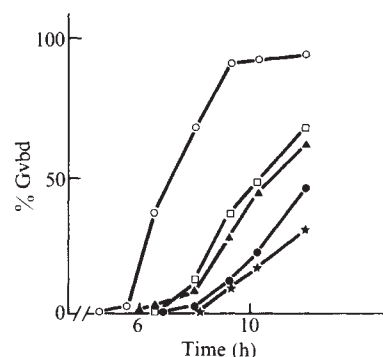
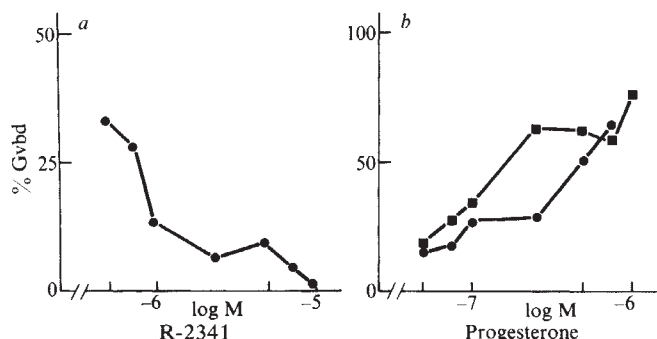


Fig. 5 Inhibition by different steroids of the progesterone-dependent release of meiosis in the oocytes of *Xenopus laevis*. The data represent the % of Gvbd, of 100 oocytes incubated at room temperature in 10 ml Barth medium (pH 7.4) plotted as a function of time after exposure to progesterone 1 μ M (○). The oocytes were all taken from the same female and preincubated for 16 h with: ethinyloestradiol, 10 μ g ml⁻¹ (□); 16 α -methyl-19-nortestosterone, 10 μ g ml⁻¹ (▲); 16 α -ethynyl-19-nortestosterone, 10 μ g ml⁻¹ (●); R-2341, 10 μ g ml⁻¹ (★).

Oestradiol attached to the polymer (up to 1 mM) and the polymer itself did not provoke Gvbd, confirming the specificity of action of the progesterone macromolecular analogue. The possibility of cleavage of the macromolecular derivative with release of active steroids during the incubation was ruled out using radioactive derivatives. These studies showed negligible uptake of the labelled macromolecular progesterone analogue compared to that of free steroids and there was no correlation between uptake and effect on Gvbd. Injection of the polymer-linked progesterone into the oocytes did not induce maturation. Finally, we found that the efficiency of the macromolecular steroid is linearly correlated with the time of actual exposure, in contrast to the curve obtained with progesterone (Fig. 6); this may be due to the hydrosolubility of the derivative, which is easily washed from the surface, contrary to hydrophobic progesterone, which is largely trapped by the oocyte. Concurrent with our studies, induction of meiosis was observed with deoxycorticosterone hemisuccinate coupled to aminoethylated agarose beads²⁴.

These results suggest that the steroid acts at the surface of the oocyte to initiate the meiotic maturation response.

Other inducers at the membrane level

A wide variety of nonhormonal compounds (Table 2), although active at the surface of cells, also trigger meiosis. Among these are several amphiphilic cationic drugs known to penetrate membrane phospholipids and increase the surface pressure.

The best representatives of such compounds are the β -adrenergic antagonists (for example, propranolol). Used in the millimolar concentration range (interaction of propranolol with β -adrenergic receptor has a K_D of approximately 1 nM), they display the so-called 'membrane stabilising effect' or 'local

anaesthetic activity' independently of their stereochemical configuration. It is possible that these effects are related to interaction with membrane phospholipid turnover²⁵⁻²⁹. In contrast, β -adrenergic antagonists lacking the hydrophobicity of propranolol, such as atenolol, practolol and sotalol, are not able to induce meiosis. The two latter compounds have no effects on phospholipid metabolism in other systems^{26,28,29}. It has been suggested that propranolol interaction with phospholipid constituents of the membrane provokes displacement of loosely bound membrane calcium with concomitant changes in calcium pools within the cells²⁵. It is possible, however, that propranolol

Table 2 Meiosis reinitiation by nonsteroidal drugs

Drugs	Concentration range (mM)	Meiosis reinitiation	Interaction with cellular phospholipid constituents*	Ca-ATPase inhibition*
Propranolol-like drugs				
(+) propranolol	0.5-1	+	+25,26,28,29	+30,31,32,66
(-) propranolol	0.5-1	+	+25,26,28,29	+30,31,32
Alprenolol	0.1-0.5	+	+26	
Atenolol	0.1-1	-		
Dichloroisoproterenol	1.0	+	+26	
Metoprolol	0.5-1	+		
Oxprenolol	1.0	+		
Practolol	0.1-1	-	-29	+31,66
Sotalol	0.01-10	-	-28,26	
Local anaesthetics				
Benzocaine	5	-	+67	
Dibucaine	0.5-1	+	+25,67,68	+70
Lidocaine	0.5-1	+	+67,69	+70
Procaïne	1-10	-	+67,69	+71
Tetracaine	0.5-1	+	+67,72	+70,73
Drugs acting on CNS				
Allobarbitol	1-5	+		
Amobarbitol	0.5-1	+		
Barbitol	1-5	+		
(+) butaclamol	0.5	+		
(-) butaclamol	0.5	+		
Chlorpromazine	0.5	+	+68,74,75	+70,76
Fenfluramine	1.0	+	+68	
Fluphenazine	0.2	+		
Haloperidol	<5	-		
Imipramine	0.5	+	+68	
Morphine	0.1-5	-		
Naloxone	0.1-5	-		
Nicotine	0.1-10	-		
Phenobarbitol	<5	-	+77	
Phentolamine	0.5-1	+		
Pimozide	0.5-1	+		
Theophylline	<10	-		
Drugs interacting with Ca fluxes				
A 23187	0.005	-	+78	+78
A 23187 plus Mg	0.005	+	+78	+78
Amiloride	<1	-		
Azide (Na)	<1	-		+79
Caffeine	<10	-		
Cromoglycate (Na)	<1	-		
Dantrolene	<1	-		+80,81
Lanthanum	5	+		+70
Methoxyverapamil	0.5-1	+		+70
Mg	<20	-		
Ouabain	1	-		
Phenformin	<1	-		
Quinidine	0.5-1	+		+70,74
Ruthenium red	<5	-		+82
Taurine	1	-		
TEA (tetraethylammonium-Cl)	<1	-		
Veratridine	<5	-		
Verapamil	0.5	+		+70

* Data from the literature obtained in various conditions and with different approaches.

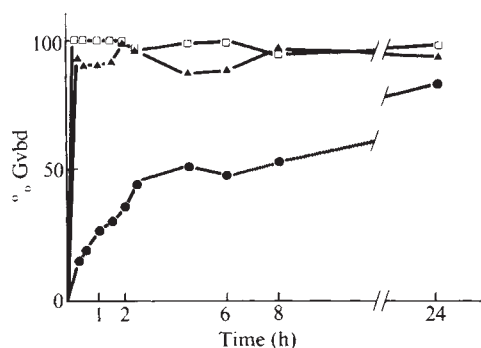


Fig. 6 Effects of progesterone and of polymer-bound progesterone on maturation (% Gvbd) as a function of the duration of hormone exposure. Batches of 100 oocytes from the same female were distributed in test tubes containing 1 ml of incubation medium. At zero time progesterone 0.1 mM ($\square$), progesterone 1 μ M ($\blacktriangle$) or polymer-bound steroid 0.1 mM ($\bullet$) were added. At various incubation times (abscissa), hormones were extensively washed with Barth medium and further incubated. After 24 h Gvbd was scored.

and amphiphilic congeners may influence intracellular Ca^{2+} levels by an action on Ca-ATPase in the oocyte membrane as has been observed in blood red cells³⁰ and in sarcoplasmic reticulum^{31,32}.

Some local anaesthetics induce meiosis, but their activity is not related to their effectiveness as local anaesthetics; thus, benzocaine and procaine are ineffective. It seems likely that the local anaesthetics act by influencing membrane phospholipids, with concomitant Ca^{2+} displacement^{25,33}, as found for propranolol-like drugs. Procaine and benzocaine have a very limited ability to displace calcium in the microsomal system³⁴.

A variety of drugs active on the central nervous system, including neuroleptics (chlorpromazine), antidepressants (imipramine), anorectics (fenfluramine) and some barbiturates, also display maturational activity. Their effect can be attributed to an interaction with membrane phospholipids (and possibly calcium) rather than with stereoselective receptors³⁵⁻³⁷. Phen-tolamine, an α -adrenergic antagonist, is active on oocytes and also shows membrane effects involving lipid metabolism and Ca^{2+} fluxes³⁸. A wide variety of drugs inactive on oocytes (Table 2) do not exhibit membrane phospholipid or Ca^{2+} effects.

Some organo-mercurial compounds³⁹, such as *p*-chloro-mercuribenzoate, have also been shown to induce maturation, possibly through interaction with SH-groups of the membrane, as induction is inversely related to their ability to penetrate the cells.

Calcium and progesterone

The earliest changes following hormone or drug interaction with the oocyte membrane leading to meiosis are unknown. However, there is considerable evidence which suggests that membrane and/or internal calcium distribution is affected.

Among drugs interacting with Ca^{2+} fluxes and/or intracellular exchanges, methoxyverapamil and verapamil were found to be potent inducers of meiosis. Both are tertiary amines and may be regarded as cationic drugs which, as propranolol-type inducers, act by displacing membrane Ca^{2+} (ref. 35), or inhibiting a Ca^{2+} efflux pump in the membrane which maintains low levels of intracellular Ca^{2+} . Inhibition of such a pump would cause the cytoplasmic Ca^{2+} concentration to rise. The positive effect of quinidine, in contrast to those of ouabain and caffeine, is probably attributable to its capacity for displacing calcium from binding sites as in the microsomal fraction of skeletal muscle³⁴. The direct or indirect interaction with Ca^{2+} fluxes of the other drugs listed in the last section of Table 2 are either not selective enough to promote meiosis, or are secondary to other ion permeability changes.

Involvement of Ca^{2+} changes has been also suggested by the action of lanthanum, which induces Gvbd and correlated

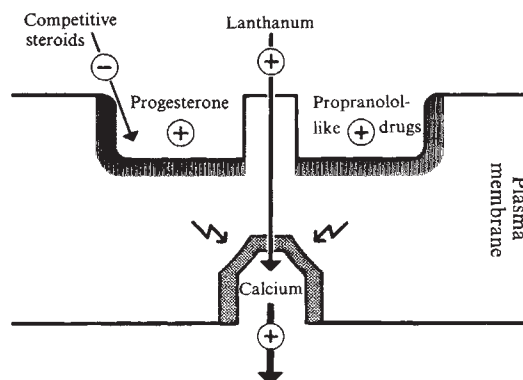
phenomena⁴⁰. La^{3+} has a very high affinity for the Ca^{2+} binding sites of Ca^{2+} -dependent ATPases. Moreover, Gvbd can be provoked by using ionophore A 23187 (if Mg^{2+} or Ca^{2+} is present⁴¹) or specific Ca^{2+} iontophoresis⁴², whereas its induction by progesterone or ionophore is suppressed when intracellular free Ca^{2+} is chelated by EGTA injection⁴³.

Calcium has been proposed as the 'second messenger' of several mitotic factors, and implicated in various changes occurring during cell division. It could then well be involved in progesterone action on oocyte meiotic division. Moreover, calcium is known to inhibit adenylate cyclase activity and its binding to phosphodiesterase activator has been reported. Indeed, early steps of progesterone action may be compatible with either a decrease of cyclic AMP and/or a decrease in the phosphorylation of protein(s) involved in the arrest of meiosis. The former has been observed in *Rana* oocytes (as well, incidentally, as the diminution of cyclic-GMP) (refs 44, 45). (However, the pattern given by our measurements of cyclic AMP in progesterone exposed *Xenopus laevis* oocytes differs somewhat from what has been found in *Rana* (S.S.-S. *et al.*, in preparation).) The latter is suggested by the effect of the injection of purified catalytic subunit of protein kinase (from rabbit skeletal muscle) which inhibits the progesterone-induced maturation⁴⁶; the regulatory subunit or the heat-stable protein inhibitor of cyclic AMP-dependent protein kinase, injected in the absence of progesterone, induces meiotic cell division. We have found⁴⁷ that cholera toxin is a powerful inhibitor of progesterone action. Taken together, these striking results suggest a role(s) for a cyclic nucleotide(s) in the meiotic cell division, possibly dependent on Ca^{2+} changes and leading to an early modification of protein phosphorylation⁴⁸ (clearly distinguishable from the burst of soluble protein phosphorylation occurring later, just before Gvbd⁴⁹).

Three membrane features

The studies reported suggest that three major areas of progesterone action at the surface level of amphibian oocytes merit detailed investigations (Fig. 7). (1) The nature of the interaction site for progesterone and other active steroids seems to be neither a conventional receptor protein nor a 'simple' nonspecific lipidic array. (2) The nature of the calcium binding site and/or ionophoric element, connected in some manner with the putative hormone site, is unknown. (3) The membrane

Fig. 7 The three membrane features implicated in the very early steps of progesterone action are schematically represented, and also seen as pharmacological targets ($\oplus$): agonist, $\ominus$: antagonist). The progesterone site can accept competing antagonist steroids (Table 1). The membrane intrinsic components can be modified by propranolol-like drugs (and other compounds interacting with membrane) at millimolar concentration (Table 2). They activate the postulated calcium site. (The drawing arbitrarily indicates that drugs do interact with membrane elsewhere than in the progesterone site: this is, however, not excluded.) The release of calcium from its membrane site follows steroidal agonist interaction in the progesterone site or drug effect on the membrane; it can possibly be provoked by La^{3+} ions.



environment is probably of importance for the activity of these hormone and calcium sites. Then perturbation of membrane structure/fluidity will alter their functions. We suggest that the various drugs of the propranolol type modify the oocyte membrane and in turn membrane-bound calcium in the same way as progesterone does.

Conclusions

Xenopus laevis oocytes have long been known to provide a useful tool for investigating several aspects of gene expression⁵⁰. Now, in relation to progesterone-induced meiotic maturation, these cells may contribute to a better understanding of other important problems.

(1) Initiation of meiosis at membrane level. Steroids are active in the resumption of meiosis in all amphibians so far tested and in lower vertebrates such as fish. In starfish, 1-methyladenine is the 'hormone'⁵¹. In mammals⁵², it was shown that one cannot release the oocyte from the follicle without resuming meiosis, and it was hypothesised that either the granular cells produce an inhibitor or that meiosis takes place in association with ovulation, which disrupts cell-cell interaction. The fundamental question is whether the processes leading to meiosis are always initiated at the membrane level and are the same in widely different species, despite differences in the initial trigger.

(2) Mechanisms of hormone actions. The molecular aspects of progesterone-membrane interaction may provide an opportunity to study a new type of target site for steroid hormones in somatic cells. For example, the very early effects of oestrogen on membrane permeability in uterine cells⁵³ may be relevant to such sites. It has already been suggested that steroid hormones act in part at the surface of target cells, and specific interactions have been described^{54,55}. The selective effects of deoxycorticosterone on *Neurospora crassa* permeability to cations⁵⁶ and of progesterone on *Dictyostelium discoideum* development⁵⁷, the specificity of steroid structure in anaesthesia^{58,59} and the physi-

cal effects of steroids on membranes^{60,61} should be further studied. In many systems, steroids are cell division and/or differentiation promoting/regulating factors, but as yet no related mechanistic studies have been carried out. The oocyte-progesterone system may suggest the importance of the interaction between steroids and plasma membrane in this respect, and more work should establish whether some features proposed in 'cell surface modulation'⁶² are involved. This is not to deny that steroid-receptor complexes act in somatic cells at the nuclear locus and that the largest part of the hormonal response is due to this action in the nucleus. The present results with oocytes support the idea that hormone interaction at non-nuclear site(s) may well be complementary to action at the nuclear level in somatic cells and may even be essential for some aspects of hormone activity (cell division). This concept, that a hormone may act at multiple loci in the target cell, has been previously discussed^{63,64}.

Finally, the possible role of calcium ions as second messenger of a steroid signal-membrane interaction is worth testing in other steroid-sensitive systems.

(3) General aspects. In *Xenopus laevis* oocytes, progesterone induces a series of events which are themselves of basic biological importance. For example, the unknown mechanism by which selective protein synthesis is involved at the translational level during progesterone action is of great interest. Mpf, a protein synthesis-dependent product of progesterone action, is remarkable because it promotes all events following meiotic division and it is possible that a similar mitotic promoting factor is to be found in somatic cells undergoing cell division under hormone stimulation.

Xenopus oocytes are arrested cells which may yield much information about events generated at the surface level to biologists who care to expose them to progesterone.

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articles

Slow earthquakes and stress redistribution

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Strainmeters with high sensitivity over long periods have enabled the detection and identification of slow earthquakes: seismic events which produce records similar to those from normal earthquakes except that the time scale for the rupture process is considerably longer. Slow earthquakes provide a mechanism for stress redistribution before normal earthquakes. Stress concentration may take place just hours or days before an earthquake; if it did, this would affect prediction capability.

EARTHQUAKES are generally assumed to result when the gradual stress buildup in a region eventually exceeds some critical local strength. The fracture which develops is thought to propagate at a velocity approaching that of shear waves. Although this is supported by laboratory experiments¹, there have always been observations which cannot be explained by fracture in an otherwise perfectly elastic medium. The time periods between events in a foreshock–aftershock sequence, for example, far exceed the time necessary for elastic redistribution of stress. Clearly, one or more other mechanisms must have a major role in the stress redistribution.

There have been various studies^{2–4} of particular earthquakes inferring coseismic processes with time scales longer than normally associated with earthquakes. However, the evidence for these so-called ‘slow’ or ‘silent’ earthquakes has generally been indirect (for example, anomalous long-period spectral behaviour³ or unusually long surface-wave coda⁴) as the time scale of such processes lies outside the passband of conventional seismographs.

A network of Sacks–Evertson borehole strainmeters⁵ was installed along the seismically active Pacific coast of Honshu south of Tokyo which enabled the investigation of the possibility of ‘infraseismic’ mechanisms for redistribution of stress. These instruments have high sensitivity from zero frequency to several Hz. In addition, tests with nearby explosions (producing accelerations up to g at the instrument) produced no spurious recordings⁵.

New, direct observations of strain redistribution interpreted in terms of events which we call slow earthquakes are reported here. All our observations are consistent with the hypothesis that these slow earthquakes are similar to normal earthquakes in

all respects except for slower rupture velocities and longer rise times. Here we describe slow earthquakes which occur separately from normal earthquakes and which were observed on the recently installed borehole strainmeters or on nearby extensometers. Other kinds of data are also included which indicate that the stress buildup before an earthquake may be non-linear in time. In these cases the concentrations of stress seem to occur in a much shorter time preceding the earthquake than that calculated on the basis of magnitude–precursor–time formulae⁶.

Strainmeter waveforms for normal and slow earthquakes

To understand the characteristics of slow earthquakes, we first examine strain waveforms from normal earthquakes, particularly in the near field (less than 10 fault lengths) where observations of slow earthquakes are most convincing. Figure 1 shows the radial strain field based on a modification of a dislocation model in the form presented by Harkrider⁷. The theory is oversimplified (for example, a point source in an infinite space is assumed) but the results are expected to be qualitatively correct.

The waveforms shown in Fig. 1 can be treated as the superimposition of two parts: a strain step which is proportional to the moment and which falls off as the cube of the epicentral distance, and a ‘radiated’ pulse which is defined here to include both a term with zero total area which falls off with distance as well as a term whose area is proportional to the moment but which falls off with the square of the distance. Within a few fault lengths (see Fig. 1a) the strain step dominates, while beyond about 20 fault lengths the step would not be detectable—except for an event with an extremely large fault offset.

Figure 2a is a wide-frequency-range borehole strainmeter record from Matsushiro of a nearby small ($m = 4$) earthquake. The compressional wave is followed by a larger shear arrival, and the static offset arrives shortly after S . The basic low-frequency structure is emphasised and the higher-frequency oscillations suppressed in the (effectively) low-pass filtered version of the same record shown in Fig. 2b; this and the record shown in Fig. 3a (a low-pass filtered record of the same earthquake from the Hokushin Observatory near Nagano) show good agreement with the theoretical strain record of Fig. 1c.

Figure 3b shows a strain event recorded by a borehole strainmeter at Irako. Although the record shown is from a

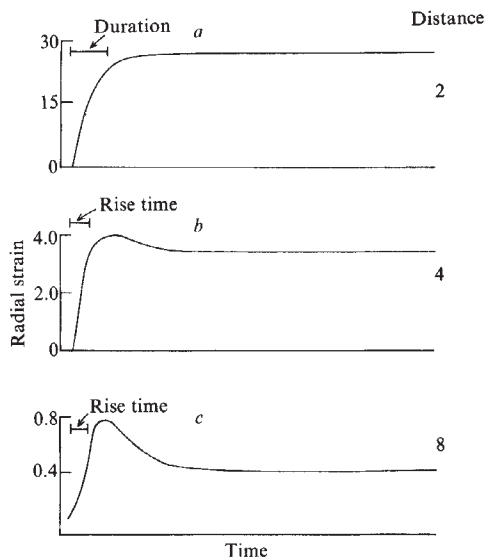
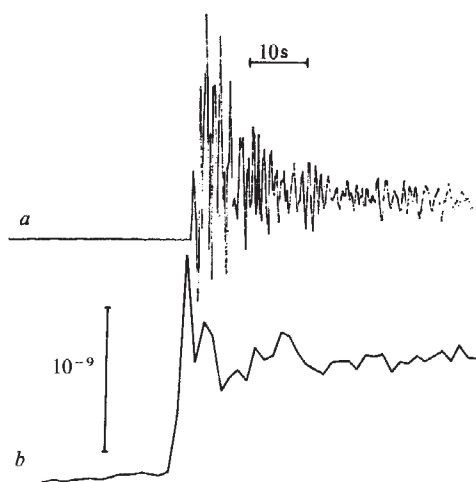


Fig. 1 Theoretical waveforms of radial strain plotted against time of an event at different epicentral distances. *a*, At close distances, there is a relatively slowly rising step. The duration is the time for which movement (rupture) occurs on the fault. *b* and *c*, at greater distances the radiated pulse is observed because it decays with distance less rapidly than does the strain step. The rise time is the time needed for the strain to achieve 86% of its peak value. The time scale is the same in all cases, and the amplitude scales with increasing distance as indicated.

wide-frequency-range channel such as that used for Fig. 2*a*, only low frequencies appear in the arrival. The waveform is similar to that of the earthquake in Fig. 3*a* and to the theoretical waveform in Fig. 1*c*. Many such events have been observed on the recently installed borehole strainmeters as well as on some nearby extensometers. The radiated pulses and strain steps from such events seem to be similar to those from normal earthquakes except that the rise time and duration are about 100 times longer than those for earthquakes of similar moment. It is therefore possible to produce slow-earthquake waveforms which are in reasonable agreement with observation by using normal-earthquake theory with long rise times and low rupture velocities.

Fig. 2 Record from the borehole strainmeter at MAT of an $m = 4.0$ earthquake on 19 September 1973 at a distance of 15 km. *a*, Broadband-response record. *b*, Low-pass filtered record from a different sensor in the same instrument. This sensor has a higher noise level⁵. The magnification of the trace in (*a*) is about one-half that of the trace in (*b*). The traces are slightly offset in time for ease in viewing.



At smaller distances, the theory then predicts records such as shown in Fig. 1*a*. Figure 3*c* shows a slow earthquake with similar waveform. It should be kept in mind, however, that for observations on only a single instrument there is an unresolvable ambiguity between an event being near, slow and of a certain moment or further, slower and of a larger moment.

Events such as those in Fig. 3*b, c* differ from creep events (observed close to a long fault) in that in such events there is no permanent change in the strain field⁸.

The expanded time scale for slow earthquakes means that almost no energy is radiated in the normal seismic frequency band, so that slow earthquakes cannot be detected by seismometer networks used for earthquake location. Even with instruments of high magnification at low frequencies, only nearby events can be clearly identified because theoretical considerations suggest⁹ that the low rupture velocities for these events result in low radiation efficiencies. Hence, such instruments are required in denser networks than now exist to do multi-instrument correlations of individual slow earthquakes.

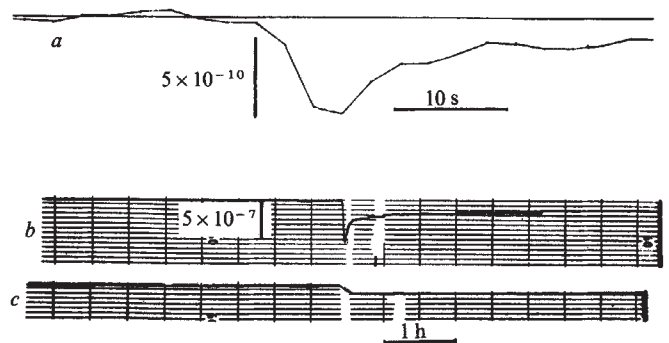


Fig. 3 *a*, Low-pass filtered strainmeter record from the same earthquake as in Fig. 2 recorded at Hokushin, near Nagano (distance = 15 km, Hokushin-MAT distance = 13 km). This record can be compared with the theoretical waveform in Fig. 1*c*. *b*, Slow earthquake record for 25 June 1976 from the broadband-response sensor of the borehole strainmeter installed at Irako, on the Pacific coast. Note the similarity between this record and that from a normal earthquake (above) as well as the theoretical waveform in Fig. 1*c*. *c*, Slow earthquake record on 26 July 1976 from Irako. This event is either of similar time scale but at a closer distance compared to the one above or it is slower and at a similar distance.

Foreshocks and aftershocks of slow earthquakes

Shallow earthquakes may have foreshocks and aftershocks which are considerably smaller than the main event. Slow earthquakes may exhibit similar behaviour; Fig. 4 shows a slow earthquake accompanied by a foreshock-aftershock sequence. Relative to the step size, the foreshocks have larger radiated pulses than the aftershocks. If these are all at more-or-less the same distance and have similar rise times, one interpretation is that the foreshocks have higher stress drops. Such a phenomenon has been claimed for some instances for normal earthquake foreshocks and aftershocks¹⁰.

Slow earthquakes as precursors for a normal earthquake

Figure 5 shows the relative locations of the borehole strainmeter at Shizuoka, the Fujigawa extensometers and the epicentre of an $m = 5.5$ earthquake which occurred on 15 June 1976. Also shown are the dates and (relative) amplitudes of strain steps associated with slow earthquakes observed at the two observatories. The Fujigawa extensometers have operated for several years, but slow earthquakes were recorded only during the period January-May 1976. The borehole strainmeter at Shizuoka began operation in early April 1976 and recorded slow

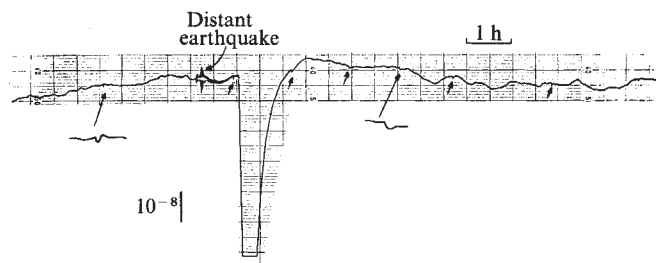


Fig. 4 Irako borehole strainmeter record for 25 August 1976. A slow earthquake, strain = 7×10^{-8} , is shown preceded by foreshocks and followed by aftershocks with strains less than 10^{-9} . Expanded-scale tracings of sample foreshocks and aftershocks are included. The strain record has been high-pass filtered at 25-min period.

earthquakes (of the opposite polarity from those at Fujigawa) from that time until early June. None of the events is recorded at both observatories. Based on the Fujigawa records, the slow earthquakes started about 150 d before the earthquake, which is an often quoted⁶ precursor time for an earthquake of that magnitude.

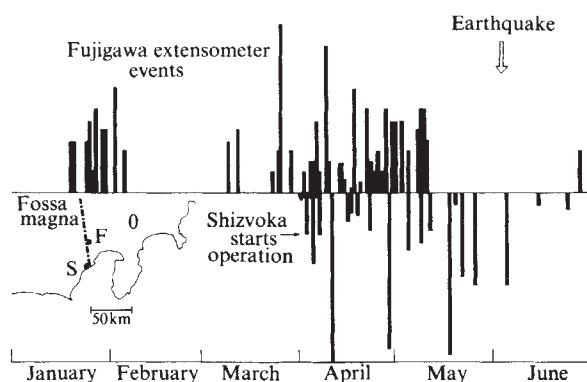
A model consistent with these observations has been suggested by Y. Okada (personal communication). The observatories are on opposite sides and within a few kilometres of the Fossa Magna, a major geological discontinuity (see Fig. 5). Okada notes that the relative amplitudes and polarities of the slow earthquakes at the two observatories could be explained if the slow earthquakes occurred on the Fossa Magna.

Other detections of preseismic stress redistribution

There are other data which indicate that the concentration of stress in an epicentral region may be very nonlinear in time: three examples are given here.

Figure 6 shows the tide-gauge record from Irozaki, on the tip of the Izu peninsula, Honshu, Japan for the day on which a nearby $m = 6.9$ earthquake occurred. In this region, the return time for such an earthquake is many decades. As can be seen in Fig. 6, there is a deviation from the expected tide level apparent about 20 min before the earthquake. This deviation is in the opposite sense to the eventual level change due to the earthquake. An interpretation could be that, due to a nearby slow earthquake shortly before the normal earthquake, there was a buildup of strain of about one-third of that eventually released.

Fig. 5 Time history of the slow earthquakes (recorded on the borehole strainmeter at Shizuoka and on the Fujigawa extensometers) preceding the $m = 5.5$ Yamanashi earthquake. The Shizuoka observatory began operation in April 1976. The amplitude scales for the two instruments are not the same, but the relative polarity of the strain steps is as indicated. Also included is a map showing the relative locations of the two observatories, the epicentre (○) and the Fossa Magna.



Rikitake¹¹ describes a number of tilt changes that have been reported preceding large earthquakes. While his examples establish that large changes of ground level or tilt occurred some days or hours before earthquakes, in most cases there are no instrumental observations which would allow quantitative estimates. One exception is the Tonankai earthquake ($m = 8$) of 7 December 1944.

Levelling surveys in the region of Kakegawa (~150 km north east of the eventual epicentre) showed slight subsidence corresponding to a tilt of 3×10^{-6} rad in the 10 yr preceding the earthquake. On the morning of the earthquake (which occurred at 13 36 LT) a level-survey team was operating north of Kakegawa. Over a 2-h period (up to 12 noon) closure errors of 9 mm over 2 km were found; during this brief period the ground had tilted 4.5×10^{-6} rad in the sense of uplift at Kakegawa. Comparison of a survey immediately after the earthquake with one completed a month before showed a tilt of about 10^{-5} rad in the region north of Kakegawa. This total tilt was in the same

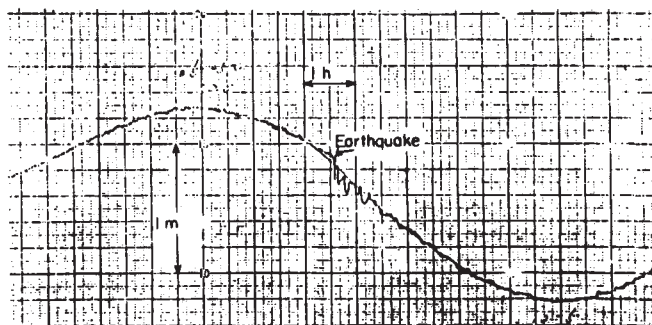


Fig. 6 Tide-gauge record from the tip of the Izu peninsula for 9 May 1974. On this date an $m = 6.9$ earthquake occurred within a few kilometres of the tide gauge. Note that preseismic motion is apparent about 20 min before the earthquake.

sense as the rapid precursory tilt on the morning of the earthquake. Thus this precursory tilt and that due to the earthquake were approximately equal.

A possible interpretation is the precursory tilt was due to a slow earthquake of moment comparable to the normal earthquake.

An $m = 5.2$ earthquake near Hollister, California, on 28 November 1974 was preceded by tectonomagnetic effects¹² and anomalous tilting¹³. Magnetic field changes of about 1.5–2 nT measured at some 16 km from the eventual epicentre occurred over a period of about 2 weeks ending 4 weeks before the earthquake. The time periods involved were much less than the normally found precursory time for an $m = 5.2$ event, and at the distance of the observing stations, there is no anomaly coincident with the quake.

These observations could be explained by slow earthquakes redistributing the regional stress in a manner similar to that suggested by Smith and Johnston¹².

On 14 January 1978, there was an $m = 6.5$ earthquake at 23 km depth near O-Sima Island, Japan. The epicentre was ~33 km from the borehole strainmeter at Irozaki. From the time of its installation in April 1976 through November 1977 the observed strain rate was less than 10^{-7} per month. Six weeks before the earthquake, a compressional excursion of 2×10^{-6} occurred over a period of about 20 d. The strain remained relatively constant at this level until about 4 d before the earthquake, when a dilatational trend began. This provides another example of stress redistribution directly preceding an earthquake.

Conclusions

It is likely that strainmeters and tiltmeters, of adequate stability and sensitivity, installed in active tectonic regions would and

have recorded similar events to the slow earthquakes reported here¹⁴.

The simplest model of events leading to an earthquake is that stress builds up gradually in the eventual earthquake region due to some process such as plate motion. At some stage of stress—and in some areas only—dilatancy may occur. In principle, it is possible to detect this dilatancy through measurements of V_P/V_S , elevation changes and other associated phenomena.

We believe, however, that this is not necessarily the sequence for all large, shallow earthquakes. The available data suggest that while there may be a stress buildup in large regions due to tectonic processes, the stress may not become concentrated in the eventual earthquake region until shortly before the earthquake. One mechanism for this nonlinear in time stress concentration is the slow earthquake.

Two significant implications are: (1) if high stresses may not be concentrated in the source region until possibly a few days or less before even large earthquakes, this would set the effective upper limit to the warning period that a specific prediction could

provide for such events. (2) If there are great slow earthquakes, this would be important in the excitation of the Chandler wobble^{15,16}.

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Aquatic sources and sinks for nitrous oxide

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Oxidation of organic material seems to represent a source for N₂O in a wide variety of aquatic systems. Consumption of the gas, attributed to microbial respiration, is observed for a few specific anoxic environments.

NITROUS OXIDE plays an important part in stratospheric chemistry^{1–4} and may influence the radiative budget of the troposphere⁵. Concern has arisen recently^{6,7} that human activity could lead to a significant increase in the source strength for atmospheric N₂O. Assessment of this possibility is hampered by major gaps in our understanding of processes which regulate N₂O in the natural system.

The gas is thought to originate primarily as a product of microbial metabolism^{8,9}. Stratospheric photolysis can remove at most 10⁷ tons of nitrogen per yr and represents the only currently established atmospheric sink^{9,10}. The magnitude of the global source is uncertain, with particular controversy attached to the role of the ocean.

Hahn¹¹ (also Hahn and Junge¹²) concluded, from limited measurements of dissolved N₂O, that the ocean should represent a major source for atmospheric N₂O, contributing as much as 10 times the quantity of gas consumed in the stratosphere. McElroy *et al.*¹³ argued, from consideration of marine productivity, that the oceanic source should be small and raised the possibility that microbial respiration in conditions of low oxygen could offer an important sink for marine N₂O. They speculated that consumption of N₂O might exceed production on an integrated basis for the world's oceans.

This article summarises recent results which attest to the complexity of the aquatic nitrogen cycle as it affects N₂O. We present data from Central and South East Tropical Regions of the Pacific Ocean, and from Chesapeake Bay. Our results support the view^{11,14,15} that oxidation of ammonium and amino nitrogen, nitrification, should represent a major source for marine N₂O. They allow a quantitative estimate for the relevant source strength, implying a global yield for oceanic N₂O of less than about 10⁷ tons N yr⁻¹. Data from the South-east Tropical Pacific and from Chesapeake Bay demonstrate consumption of dissolved N₂O in conditions of low oxygen, as observed by others^{15,16}. There is no evidence to date, however, for consumption of atmospheric N₂O by the open ocean. We report preli-

minary observations indicating consumption of atmospheric N₂O by a number of aquatic systems, including a freshwater pond and a tidal saltmarsh.

Central Pacific

The data discussed here were collected between 2 and 18 December 1977 on the Norpax Leg II cruise of the RV Kana Keoki. Samples were taken to depths of 1,000 m every degree of latitude along the 150° W meridian, between 17° S and 20° N, and were analysed for temperature, salinity, O₂, N₂O, NO₂⁻, NO₃⁻, NH₄⁺, PO₄³⁻ and SiO₂. Analytical techniques are discussed elsewhere^{17,18}.

Cross sectional contours for N₂O, PO₄³⁻ and NO₃⁻ exhibit several similar structures (Fig. 1). Highest concentrations of N₂O are observed at 9° N at a depth of 129 m in an intense feature associated most probably with upwelling in the north tropical divergence zone¹⁹. This feature may be seen also in the contours for PO₄³⁻ and NO₃⁻. Highs in N₂O at 17° N (128 m) and 4° S (502 m) are similarly correlated with structure in PO₄³⁻ and NO₃⁻.

Concentrations of N₂O in surface waters are generally close to equilibrium with the atmosphere. Largest surface concentrations are observed in the zone of high productivity associated with the equatorial upwelling, where N₂O is supersaturated with respect to air by a factor of ~1.4.

On average, surface waters are supersaturated with respect to the atmosphere by a factor of ~1.25 (±0.1), equivalent to an excess of N₂O equal to 38 ± 10 ng N l⁻¹. The uncertainty in this quantity reflects imprecision in the available solubility data, errors associated with the application of these data to seawater, and inevitable uncertainties introduced by subtraction of nearly equal experimental quantities.

Highest concentrations of N₂O in Fig. 1 correspond to regions of low oxygen. It is of interest to consider the relationship between ΔN₂O and ΔO₂, where ΔX defines the excess of X with respect to the atmosphere at the local water temperature and salinity. The quantity ΔN₂O provides a measure of the net accumulation of N₂O in a water mass since its last contact with the air. By the same token -ΔO₂ provides a cumulative record of the net quantity of oxygen consumed over the same period due to oxidation of organic material.

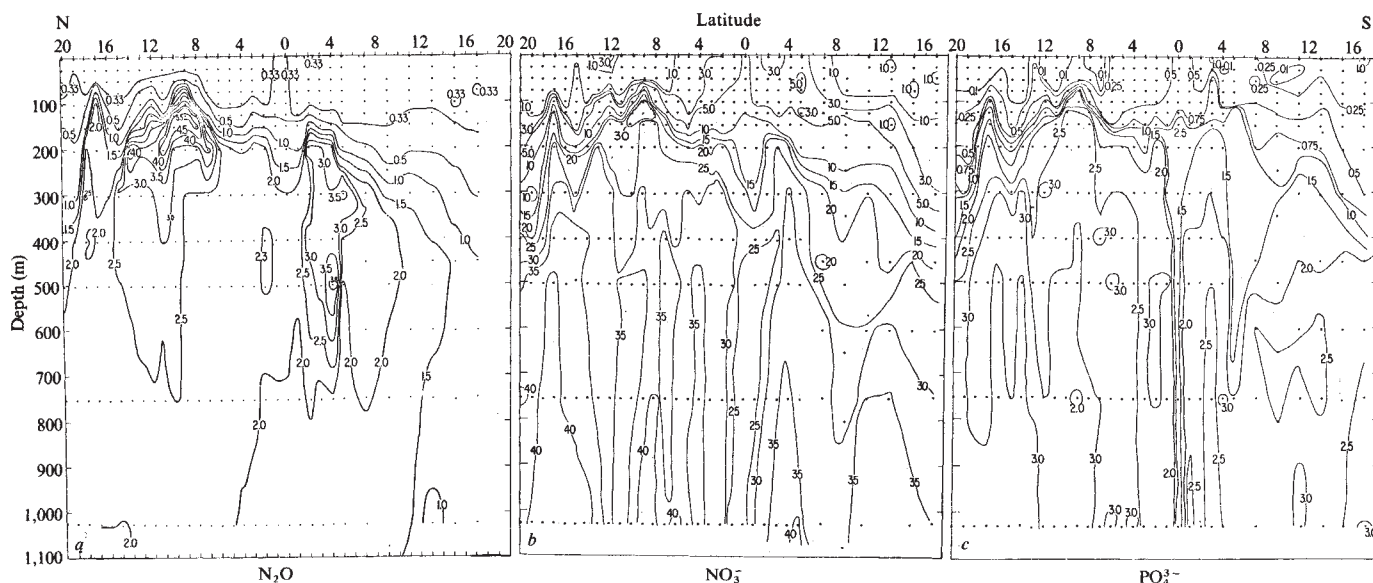
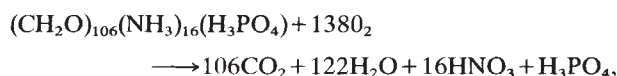


Fig. 1 Cross sections for *a*, N_2O ($\mu\text{g l}^{-1}$); *b*, NO_3^- and *c*, PO_4^{3-} ($\mu\text{g-atm l}^{-1}$) along the 150°W meridian in the tropical Central Pacific Ocean. Experimental precision is better than 3% for N_2O and $0.1 \mu\text{g-atm l}^{-1}$ for NO_3^- and PO_4^{3-} .

Figure 2a shows a plot of $\Delta\text{N}_2\text{O}$ against $-\Delta\text{O}_2$ (the apparent oxygen utilisation or AOU). We include here all of the data in Fig. 1, except for results from active regions at 17°N and $8-13^\circ\text{N}$ (above 200 m) and from 2 to 4°S (above 500 m). The results indicate (see also ref. 14) that a linear relationship may exist between $\Delta\text{N}_2\text{O}$ and $-\Delta\text{O}_2$, and that this relationship may hold over extensive regions of the world's oceans. Indeed data from active zones may also exhibit linear dependence of $\Delta\text{N}_2\text{O}$ on $-\Delta\text{O}_2$ (Fig. 2b). The value for $\Delta\text{N}_2\text{O}$ is, however, larger in this case for any particular choice of $-\Delta\text{O}_2$.

The linear behaviour exemplified by Fig. 2 suggests that production of N_2O , at least for portions of the ocean below the mixed layer, may occur primarily as a byproduct of the oxidation of organic material¹⁴. Production of N_2O during nitrification, observed in culture for both soil²⁰ (*Nitrosomonas europaea*) and marine (*Nitrosococcus oceanus*) organisms (unpublished data from this laboratory) offers a plausible explanation. If we assume that the C:N:P ratios in marine organic material are similar to values quoted by Redfield *et al.*²¹, 106:16:1, and assume further that the stoichiometry of decay may be represented by



then the data in Fig. 2 may be used to estimate the corresponding yield for N_2O . The behaviour exhibited in Fig. 2a suggests that 1 molecule of N_2O may be formed on average for every 4,700 carbon atoms which undergo oxidation.

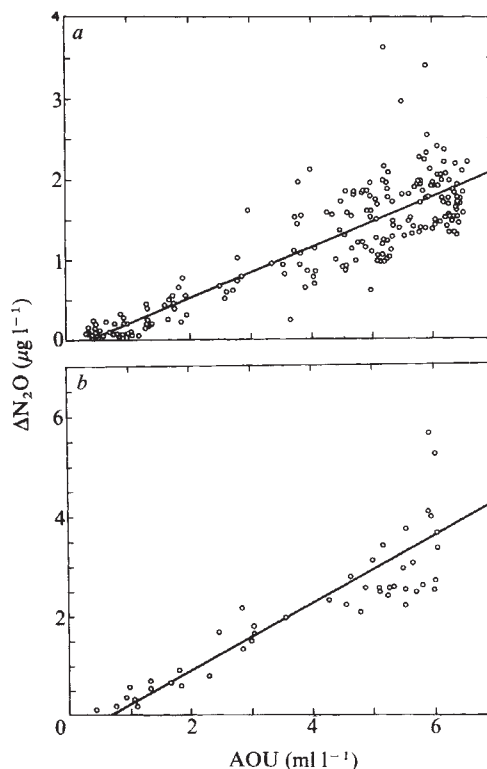
The difference in slopes exhibited by the data in Fig. 2a and b might be attributed to various factors—differences in the C:N:P ratios of organic material; intrinsic differences in the yield for production of N_2O ; *in situ* production and loss of O_2 due to photosynthesis and respiration; complications due to admixture of different water masses; and additional sources of N_2O in active regions associated either with denitrification or with assimilatory reduction of nitrate. It seems that N_2O may be an increasing function of temperature at given AOU. The relation

$$\Delta\text{N}_2\text{O} = -0.46 + \{0.180 + 0.027T\} \text{ AOU}, 25 > T > 5^\circ\text{C}$$

with $\Delta\text{N}_2\text{O}$ and AOU measured in units of $\mu\text{g l}^{-1}$ and ml l^{-1} respectively and with T in $^\circ\text{C}$, provides an excellent fit to all of the data except results from the active region between 8 and 11°N . The root mean square deviation of the data from values

predicted on the basis of the above relation is only $0.3 \mu\text{g l}^{-1}$ for 238 observations characterised by an average value of $\Delta\text{N}_2\text{O}$ equal to $1.4 \mu\text{g l}^{-1}$. The higher yield for production of N_2O at elevated temperature is consistent with temperature sensitivities observed in the laboratory by Yoshida and Alexander²⁰.

Fig. 2 N_2O observations (Fig. 1) expressed as excess N_2O plotted against apparent oxygen utilisation. Most of the data are shown in (a) but data from the active regions at 17° , $13-8^\circ\text{N}$, and $2-4^\circ\text{S}$ are plotted separately in (b). The straight lines are least-squares fits, which yield r.m.s. deviations of 0.37 and $0.6 \mu\text{g l}^{-1}$ for (a) and (b) respectively. Data points from the mixed layer ($\text{AOU} < 0.3 \text{ ml l}^{-1}$) are not included. The measured concentration of atmospheric N_2O was 291 parts per 10^9 (by volume) (p.p.b.).



Peruvian upwelling

An extensive series of measurements was carried out off the coast of Peru from the RV Knorr between 20 and 31 March 1978. A representative depth profile, including data for temperature, N_2O , O_2 , NO_2^- , NO_3^- and NH_4^+ , is given in Fig. 3. For comparison, concentrations of N_2O expected on the basis of the temperature dependent formula quoted above are shown.

The empirical relation provides excellent agreement with N_2O measurements below 300 m and is in accord also with observations at 30 and 50 m. A major discrepancy exists between 100 and 300 m. Comparison of observed and calculated profiles suggests that consumption of N_2O may be important in this zone. Indeed the observed concentration of N_2O is undersaturated with respect to the atmosphere between 150 and 250 m, a region in which oxygen concentrations lie below 0.1 ml l^{-1} .

The zone of low O_2 and N_2O includes a maximum in the concentration of NO_2^- , a feature attributed by others^{15,22} to reduction of NO_3^- . The minimum in N_2O almost certainly reflects respiration of the gas at low concentrations of O_2 as suggested by McElroy *et al.*¹³ and by Cohen¹⁶. It is consistent with a model²² which invokes a major role for denitrifying bacteria at concentrations of oxygen below 0.1 ml l^{-1} .

Further support for the role of denitrifying bacteria may be inferred from incubation experiments carried out as part of the present sequence of observations. Most samples for N_2O analysis were killed immediately with $HgCl_2$. A number of samples, however, were incubated live, in 60 ml BOD bottles. Some of these samples were treated with trace concentrations of C_2H_2 , a procedure known²³ to inhibit reduction of N_2O by denitrifiers. Samples of water from regions of low O_2 , treated with C_2H_2 , showed generally high concentrations of N_2O following 18-day incubation intervals. In one case a sample, with an initial concentration of N_2O equal to $1.3 \mu\text{g l}^{-1}$, developed a concentration of N_2O of $4.3 \mu\text{g l}^{-1}$ within an hour of application of C_2H_2 . The concentration of N_2O had reached $191 \mu\text{g l}^{-1}$ after 18 days, indicating accumulation of N as N_2O in an amount equivalent to $\sim 30\%$ of the starting concentration of NO_3^- . Results from eight other C_2H_2 treated incubation experiments showed 18-day N_2O concentrations in the range 3.3 to $47.0 \mu\text{g l}^{-1}$. Net consumption of N_2O was exhibited by all samples incubated without C_2H_2 .

Other systems

Figure 4 summarises N_2O observations taken in Chesapeake Bay during July 1977. The data were acquired in waters with total depths in excess of 13 m, and show clear evidence for consumption of N_2O below the seasonal thermocline.

There appears to be an abrupt change in the behaviour of N_2O when the concentration of O_2 falls below 1.4 ml l^{-1} . Waters are then undersaturated in N_2O with respect to air. Indeed at several stations, concentrations of both O_2 and N_2O were observed to lie below present detection limits of 0.1 ml l^{-1} and $0.02 \mu\text{g l}^{-1}$ respectively.

Fig. 3 Depth profiles for N_2O , mineral nitrogen, oxygen and temperature for a station at $15^\circ 10.6' \text{ S}$, $75^\circ 35.5' \text{ W}$, in the region of Peruvian upwelling. The dashed line represents the relationship $\Delta N_2O = -0.46 + 0.180 + 0.027T \text{ AOU}$.

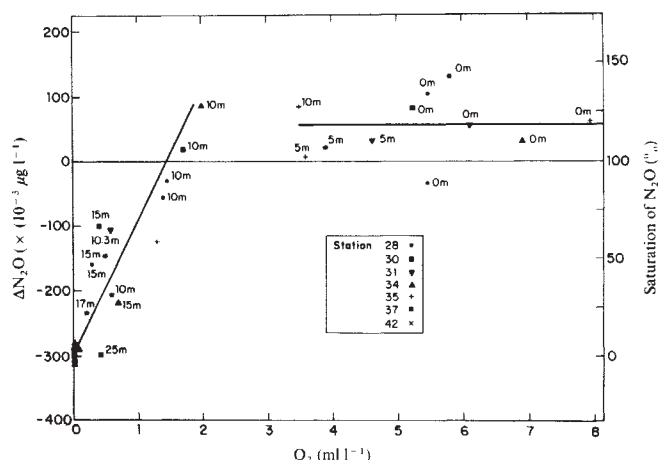
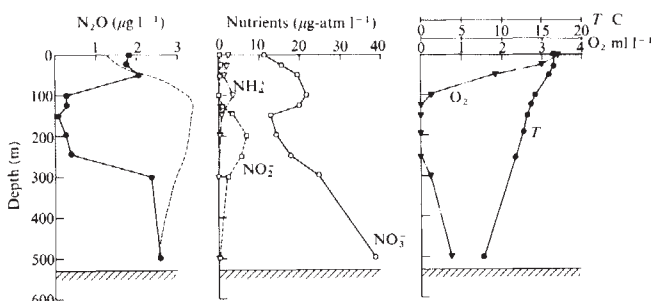
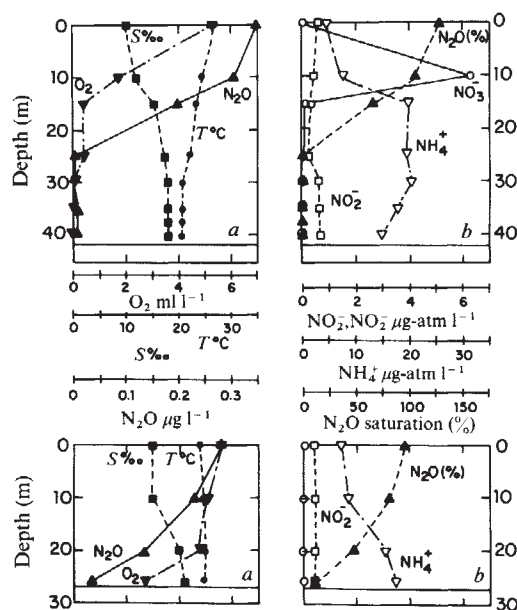


Fig. 4 N_2O and O_2 data for Chesapeake Bay. The % saturation of N_2O is given relative to the observed atmospheric concentration of 291 p.p.b., for $T \approx 27^\circ \text{C}$.

Depth profiles for N_2O , O_2 , temperature, salinity and mineral nitrogen are shown for two stations in Fig. 5. Station A, included in Fig. 4, illustrates the rapid decrease with depth below the thermocline observed for both N_2O and O_2 . Note that NH_4^+ is the dominant form of mineral nitrogen at all depths in these waters. Station B, occupied following a period of cool windy weather, shows evidence for transient breakdown of the thermocline, with associated mixing of deep and shallow waters. Note in this case that surface waters seem to be undersaturated in N_2O with respect to the atmosphere, suggesting that in summer the Chesapeake may represent at least a transient sink for atmospheric N_2O following periods of rapid vertical mixing. A more extensive sink may occur in anoxic muds associated with tidal saltmarshes as indicated by preliminary studies of a marsh on the margin of the bay at the Choptank River. Waters draining this marsh at low tide were undersaturated in N_2O by 30% with respect to air.

Fig. 5 Depth profiles for N_2O , O_2 , T and S in Chesapeake Bay for station (a), occupied 14 July 1977, and nearby station (b), occupied 27 July 1977. Weather was generally warm and stable, except for a cool windy period 25–26 July. Note undersaturation of N_2O in surface water and nearly isothermal structure at station (b). Location of (a) is $38^\circ 50' 05'' \text{ N}$, $76^\circ 23' 53'' \text{ W}$; location (b), $38^\circ 40' 15'' \text{ N}$, $76^\circ 25' 00'' \text{ W}$.



Consumption of N_2O seems to arise for at least one other system at oxygen concentrations below 1.4 ml l^{-1} . Figure 6a shows a summary of results obtained in a diurnal study of a shallow pond (denoted pond A) in Concord, Massachusetts, a system reported elsewhere¹⁷ to represent a sink for atmospheric N_2O . The present study confirmed that this pond was undersaturated in N_2O over a complete diurnal cycle. Concentrations of O_2 are relatively high during the day, reflecting a strong photosynthetic source. Concentrations of N_2O increase with time when O_2 concentrations are above 1.4 ml l^{-1} , reflecting, presumably, infusion of gas from the air. Figure 6 gives clear evidence for consumption of N_2O at oxygen levels below 1.4 ml l^{-1} . In contrast to pond A, the adjacent pond B remains well oxygenated through the night. It functions as a source of atmospheric N_2O and exhibits a relationship between N_2O and O_2 similar to that observed in the Central Pacific (see Fig. 6b).

It is probable that the sinks observed for N_2O in both the Chesapeake bottom water and in the pond discussed above, reflect utilisation of the gas as an electron acceptor by micro-organisms functioning at low levels of oxygen. Alternate elec-

tron acceptors, such as NO_3^- and NO_2^- , are present in relatively low concentration in these systems, in contrast to the Peruvian upwelling regions where NO_3^- and NO_2^- are relatively abundant. It would be tempting to attribute consumption of N_2O in all these systems to denitrifying bacteria. The hypothesis that denitrifying bacteria may effect removal of N_2O is supported for Peru by the inhibition experiments described earlier. Further work is necessary to clarify the nature of the sinks operative in the pond and bay.

Conclusions

Data from the Central Pacific, Peru, Chesapeake Bay and various ponds and rivers demonstrate that aquatic systems contain both sources and sinks for N_2O . Nitrification may represent a dominant source for N_2O in both freshwater and saltwater systems. Observations from the Pacific, elaborated above, and from various rivers and ponds^{17,18} (see also Fig. 6b) support this conclusion. Present observations suggest that approximately 1 mol of N_2O is formed for every 700 mol of NH_4^+ which undergo oxidation.

A similar yield was observed in the laboratory for soils at comparable pH by Bremner and Blackmer²⁴, suggesting that this yield may have wide application. Indeed nitrification could represent a dominant global source for atmospheric N_2O .

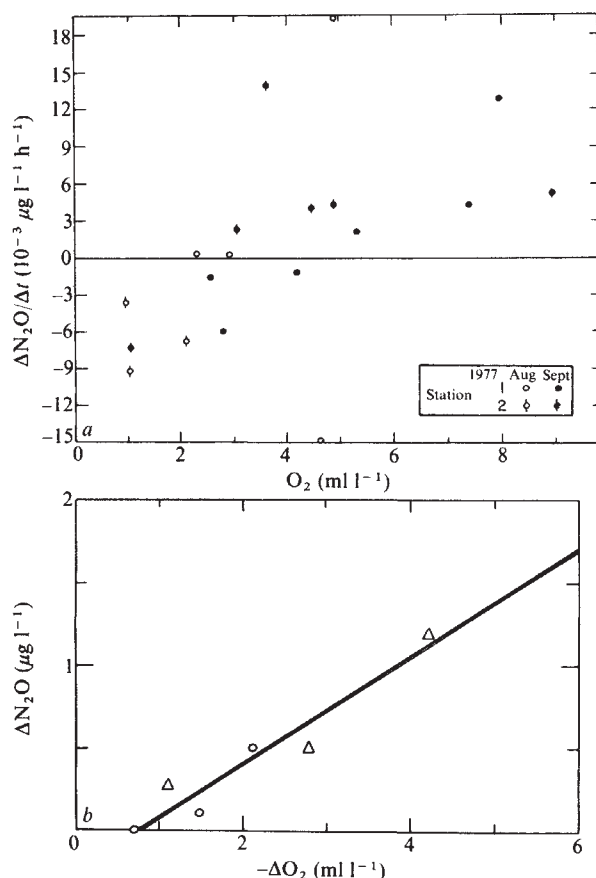
The marine contribution to atmospheric N_2O should not exceed $10^7 \text{ tons N yr}^{-1}$. This follows from the assumption of a C:N ratio equal to 6.6 for the marine biosphere²¹. It applies if the rate for marine fixation of carbon^{25,26} is $2 \times 10^{10} \text{ tons C yr}^{-1}$ and if one can ignore assimilation of reduced forms of N by phytoplankton. A source of $\sim 10^7 \text{ tons N yr}^{-1}$ is consistent with most of the available marine data and is in accord with the Pacific surface measurements discussed here.

As discussed by McElroy *et al.*¹³, it is difficult to derive a reliable value for the global source strength of N_2O based on limited data from surface ocean waters. However, applying the conventional procedure^{11,12} to our data for the surface waters of the Central Pacific gives an estimate of a marine source for atmospheric N_2O of $7 \times 10^6 \text{ tons N yr}^{-1}$ between latitudes $\pm 20^\circ$. To make this estimate one must assume negligible time variability in the concentration of dissolved N_2O in surface water, zonal uniformity in the excess of its concentration with respect to the atmosphere, and a constant piston velocity. From our own data (Figs 1 and 3) it seems unlikely that these assumptions apply to the world oceans, although the resulting estimate is coincidentally close to the upper limit provided by the oxidation model. The multiple equilibration procedure used here provides from each analysis a measure for the solubility of N_2O in seawater. Our data suggest a solubility perhaps 10% larger at 25°C than that deduced from ref. 27 and would imply therefore that the source estimated from the thin-film model should be reduced by 30%. Note that the final concentrations of dissolved N_2O deduced from our analyses are independent of the solubility coefficient for the gas.

Microbial respiration in conditions of low oxygen can represent a sink for atmospheric N_2O . The importance of the process on a global scale is not clear. It has been demonstrated here only for relatively small scale environments. Results from the Chesapeake marsh are intriguing, however, in that coastal marshes and wetlands occupy significant areas of the globe, about $2 \times 10^6 \text{ km}^2$, comparable to areas occupied by regions of intensive coastal upwelling. Further studies are obviously required to quantify the part which biological uptake may play in the global budget of N_2O .

We acknowledge the hospitality of Norpax (North Pacific Experiment) and we thank K. Wyrski, B. Patzert, T. Clarke, E. D. Stroup, S. Watson and G. A. Berberian for their contributions. We are indebted to J. Ledwell, L. Hashimoto and A. P. Durán for useful discussions. This work was supported by the US NSF and NASA under grant ENV76-24239 and contracts NASW-2952 and NSG 2031 respectively to Harvard University.

Fig. 6 a, Time rate of change for the concentration of N_2O in Concord pond A, shown as a function of dissolved oxygen. The pond is about 0.4 m deep and is stagnant, with no surface outlet. The water contained abundant flora but had negligible mineral nitrogen and was undersaturated in oxygen and nitrous oxide throughout the experimental period. b, Variations of N_2O and O_2 for Concord pond B. Quantities plotted represent differences between concentrations measured during the night, and concentrations measured at sunset, a procedure adopted to avoid confusion due to photosynthetic production of oxygen. Pond B contained appreciable dissolved NO_3^- ($\sim 10 \mu\text{g-atm l}^{-1}$). It is located adjacent to pond A and has a surface inlet and a surface outlet, with a water retention time of ~ 3 days. N_2O was supersaturated with respect to the atmosphere at all times, while concentrations of O_2 ranged between 3 and 7 ml l^{-1} . The best-fit linear relation between N_2O and O_2 is given by $\Delta N_2O = -0.235 (\pm 0.16) - \Delta O_2 \times 0.32 (\pm 0.06)$, close to the average line for all data from the Central Pacific.



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Nautiloid growth rhythms and dynamical evolution of the Earth–Moon system

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Daily growth lines and lunar monthly septa are formed in Nautilus pompilius Linnaeus shells. The number of days per lunar month determined using fossil shells has increased dramatically during the last 420 Myr, indicating that during this period the Moon revolved more rapidly and was much closer to the Earth than has previously been expected.

THE hypothesis that the Moon and Earth have been closer in Phanerozoic times has received considerable support from studies of the growth cycles of corals^{1,2}, bivalves^{3–8}, brachiopods⁹ and stromatolites^{10,11}, as well as from astronomical studies of tidal friction^{12–14}. These studies yield information about the rotation rate of the Earth, or the number of days per year, which is consistent with a closer and faster revolving Moon in earlier times. Tidal friction, by which bulges raised in the oceans by the Moon exert a torque on the Moon, implies the gradual expansion of the lunar orbit as well as an increase in the length of day with time. Both of these predictions have been confirmed for recent years by occultation measurements¹⁵, and for historical times by studies of ancient eclipses¹⁶. Unfortunately, the past dynamics of the Earth–Moon system cannot be derived solely from theoretical calculations based on recent and historical astronomical observations since tidal friction is critically dependent on sea level and continental configurations, both of which have changed markedly in the geologic past. Since an accurate charting of lunar orbital evolution has profound implications on lunar origin theories and on cosmological theories¹⁷ which predict lunar orbital expansion as a consequence of a varying gravitational constant, another approach is needed.

One viable approach to prehistoric lunar dynamics is through palaeobiology¹⁸. Nautiloids are organisms which possess a long evolutionary history (~500 Myr), but until now, they have not been investigated as possible geochronometers. We shall examine the hypothesis that there are daily and lunar monthly growth records preserved in the shells of *Nautilus*, and also in the fossil nautiloid shells beginning in the Ordovician, and discuss the implications for the evolution of the Earth–Moon

system. This synthesis suggests that the rate of lunar orbital evolution has been much greater in the past 420 Myr than short-term astronomical studies and theoretical calculations indicate.

Growth of *Nautilus*

Although nautiloid fossils are found throughout the world, the chambered *Nautilus* survives in the southwestern Pacific Ocean as the only genus of externally shelled cephalopods. Observations of *Nautilus in situ* are limited because of its nocturnal habits and its habitat—depths of up to 400 m and away from land masses. Observations on juvenile *Nautilus* are extremely limited²⁰. Thus, our study of *Nautilus* growth relies primarily on a study of shell macro and micro-structure, rather than on observations of the living *Nautilus* in its natural surroundings.

As *Nautilus* grows, convolutely coiling its shell in the shape of a logarithmic spiral (Fig. 1), it incorporates two kinds of rhythmically repeating structures. One is the secretion of the outer shell at the aperture: miniscule incrementations of calcium carbonate occur in sets of three to five, separated from preceding and succeeding sets by a ridge known as a growth line. The other rhythmic pattern is septation, by which the parts of the shell abandoned by the animal are partitioned off into chambers.

The aperture receives new growth increments at the margin, approximately half a revolution ahead of the chamber wall. The organism periodically pulls itself towards the extended aperture and deposits another septum behind it. No direct time association, therefore, exists between these two growth measures. A span of months separates the deposition of a chamber wall and the growth lines counted for that chamber. However, throughout the animal's life these septa are placed at remarkably regular intervals in the shell (Fig. 1).

As shown in Fig. 2, the number of growth lines per chamber length in *N. pompilius* is 30 ± 2 , a relationship that seems to hold throughout ontogeny. The present length of the lunar synodic month is 29.53 days. This suggests that one growth line is deposited each day and one septum is developed each lunar synodic month. While the growth rates and life habits of *Nautilus* remain to be studied extensively, the following lines of evidence suggest that this timing is reasonable. (1) Other molluscs show tidally induced or circadian skeletal banding at about the same rate of growth^{3–6}. (2) The activity cycle and

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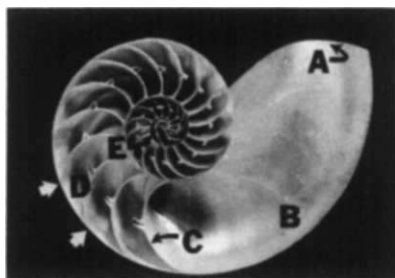


Fig. 1 Longitudinally sectioned shell of *Nautilus pompilius* showing **A**, margin of aperture where growth lines are secreted; **B**, body chamber where animal lives; **C**, septum or chamber wall; **D**, chamber space. The number of growth lines per chamber length (between arrows) can be counted using a scanning electron microscope. These counts reveal a constant number of growth lines per chamber. If our hypothesis is correct, the chambers are secreted at lunar monthly intervals.

depth migration²¹ (movement into shallow waters at night) of *Nautilus* and the attendant stress on physiology makes the expression of a daily rhythm in the skeleton seem very likely, particularly when considering the effect of periodic respiratory changes upon growth line formation²². (3) Experimentally reared cephalopods (*Sepia* and *Sepiella*) showed daily lines²³. And (4), that successive septa are formed at intervals of at least several weeks has been supported by studies of the gas content of *Nautilus* chambers^{24,25}.

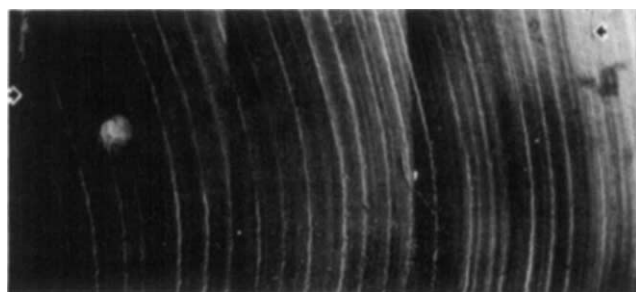
Therefore, we accept as a working hypothesis the idea that the rate of secretion of growth lines is circadian, and chambers circa-lunar. Thus, one rhythm is tied to the Earth–Sun relationship, the other to the Earth–Moon.

Growth line counts

The chambered cephalopods, including nautiloids, are probably among the most accurately dated of all fossils, due to their rapid evolution and extensive preservation in sedimentary deposits around the world. Fossil nautiloid growth lines are quite easily discerned, well preserved, and unambiguous, as shown in the photographs of growth lines (Figs 4, 5). The growth rhythm of nautiloids appears to manifest itself not just in regional settings. For example *Aturia* sp. found in Australia exhibits essentially the same growth rhythm as *Aturia curvilineata* found in Florida (Table 1). As nautiloid growth lines are believed to be daily in nature and not tide-induced, determination of the nature of the tidal cycles present in the animal's habitat is unnecessary. All of the above factors fulfill criteria essential for reliable and useful nautiloid growth line counts in geochronometry.

Growth lines are present on many superbly preserved fossil specimens. Growth lines on *N. pompilius* were examined and photographed using a scanning electron microscope or a stereoscopic optical microscope with oblique light to accentuate the growth lines.

Fig. 2 Scanning electron micrograph of the growth lines present on the outer shell of a recent *Nautilus pompilius*, ventral section. The number of growth lines per chamber (between arrows) averages 30. The number of days per lunar synodic month is 29.53.



The number of growth lines per chamber was counted on 184 different chambers of 9 recent and 29 fossil nautiloid specimens (Table 1). The specimens represent nine geologic periods and a range of about 420 Myr. Figure 1 illustrates the structure of the *N. pompilius* shell, showing the location of growth line counts. Figure 2 reveals the distinct growth lines on the ventral side. All of the counts of growth lines per chamber for consecutive undamaged chambers of recent *N. pompilius* approximated 30 (Table 1). For individual chambers, the number of growth lines ranged from 28 to 34; the average for all chambers of all specimens was 30 ± 2 . The radial lirae, or ridges, on the internal layer of the initial whorl of three *N. pompilius* specimens were counted, consistently revealing 26–32 lirae per chamber (Fig. 3), an average of 29 per chamber per specimen.

As we go back in geologic time, the number of growth lines per chamber decreases. The number of growth lines per chamber had increased from 9 at 420 Myr ago to 30 today (Table 1). Three broad conclusions can be drawn from our data. First, the number of growth lines per chamber is constant for the various chambers in a single specimen. Second, many of the nautiloids living at any given time secrete the same number of growth lines per chamber. This seems to imply a ubiquitous rate of septal and outer shell deposition at any particular geologic time. Third, and most important, the number of growth lines per chamber decreases with increasing geologic age (Fig. 6).

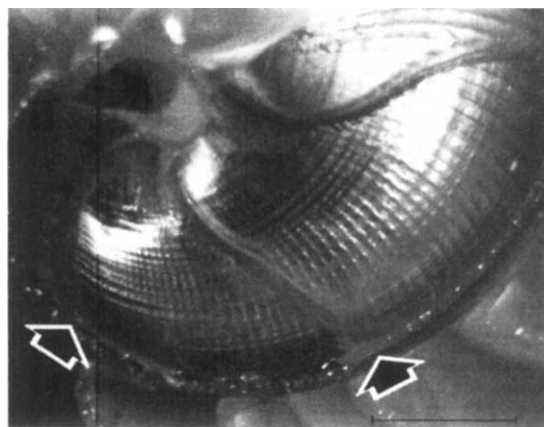


Fig. 3 Ventral section of *Nautilus pompilius* exposing the radial lirae on the interior of the chambers of the initial whorl. The lirae correspond to external growth lines, and their number/chamber averages between 28 and 30. Arrows mark chamber walls. Scale bar, 0.5 cm.

Application to Earth–Moon system

By assuming that septal secretion was controlled in the same way for fossil nautiloids (on a lunar synodic monthly basis), we can calculate the period of the Moon's orbital motion and the Earth–Moon distance up to 420 ± 10 Myr in the past, the age of our earliest specimens. The calculations made involve the following assumptions: (1) The length of the sidereal year is assumed to be constant by virtue of the large moment of inertia of the Earth around the Sun²⁶. (2) The Earth's period of rotation is assumed to have increased at the rate of 25 s per million years, the consensus result indicated from ancient eclipse studies¹⁶, some astronomical studies²⁷, and from palaeontological studies of fossil corals¹. (3) The mass of the Earth and Moon have remained constant for the past 500 Myr. We assume also that the gravitation constant is unvarying. (4) To simplify calculations, the barycentre of the Earth–Moon system is taken as the centre of the Earth.

Although the number of days in the synodic month seems to be recorded by nautiloids, for our orbital calculations the

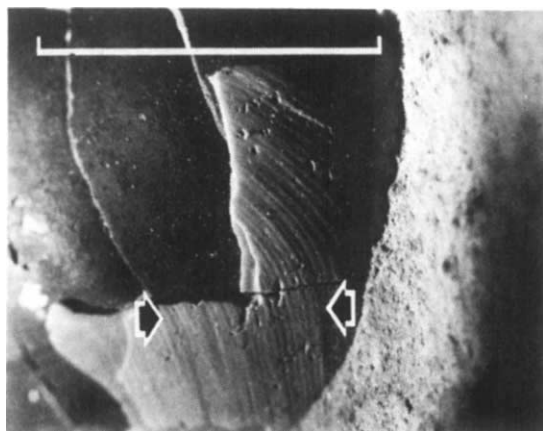


Fig. 4 Close-up of the outermost shell layer with growth lines on a remarkably preserved *Eutrephoceras dekeyi* from the Pierre Shale of South Dakota (Upper Cretaceous, ~69.5 Myr ago). The number of growth lines per chamber (between arrows) is 22 ± 2 . If chamber secretion involves a lunar monthly rhythm, then the Moon circled the Earth every 22 days, 69.5 Myr ago. Scale bar, 1 cm.

sidereal month must be used. Following Runcorn²⁸, the relationship between the synodic month S_n , the sidereal month S , and the present year length Y , can be expressed for any geologic time as:

$$S = S_n / (1 + S_n / Y)$$

To avoid confusion originating from changes in the day length, these values are expressed in hours. Applying Kepler's third law, neglecting eccentricity, yields the semi-major axis of the Moon's orbit for any geologic time.

Mode of life of *Nautilus* and nautiloids

Nautilus pompilius Linnaeus does not crawl on the ocean bottom, but instead is an active epibenthic swimmer. Several lines of evidence point to a similar mode of life for most fossil nautiloids, particularly the development of ribbing and coiling of shells and of chambers with gas and cameral deposits to maintain the body chamber in a horizontal position. This orientation would be essential for any nautiloid to propel itself effectively.

Three species, *Welleroceras liratum* Miller and Furnish, *Pseudorthoceras knoxense* McChesney, and *Orthoceras uncamera*, did not fit the general trend shown in Fig. 6. However, counts for these shells did possess integral multiples of the predicted number of growth lines per chamber length. One

species, *Pseudorthoceras knoxense*, had growth lines grouped together in threes, and the number of growth line groups was equal to the growth line counts of other specimens of that time. The evidence suggests that the modes of life of these three nautiloid species did not resemble that of modern *Nautilus*. Though depth ranges of fossil cephalopods are difficult to establish²⁹, we suggest, based on morphologic³⁰ and palaeo-ecologic studies³¹, that these three, each displaying weak septa, may have been restricted to a relatively shallow shelf and inland sea habitat (50–100 m depth). In addition, the *Welleroceras liratum* may not have been able to maintain a horizontal orientation and may have moved by crawling with its tentacles³¹. How strongly their ecologic niche was influenced by tidal interference and if this is reflected in their growth pattern is not known. Thus, specimens with relatively weak septa and/or inability to maintain the body chamber in a horizontal position could not be active deep water swimmers like *Nautilus*. Those fossil specimens of the orders Orthocerida and Nautilida that fit the growth trend are considered to have withstood similar depths as recent *Nautilus*³⁰.

The eyes of *Nautilus*, like those of other cephalopods, are quite sensitive to variations in amount of light, despite the absence in *Nautilus* of iris, lens, and eyelid³². Large numbers of cephalopods migrate upwards at night³³, and *Sepia* becomes so buoyant in darkness that it cannot help but rise³⁴. As for *Nautilus*, its activity has been reported^{21,31}, although not confirmed¹⁹, to be related to light and darkness with the animal becoming active at sunset and migrating nocturnally to lesser depths.

Whether the *Nautilus* secretes growth lines and septa during the day, when it rests in deep water, or at night, when it actively feeds, remains unknown. A theory of molluscan growth line formation based on respiratory changes²² would imply that the growth increments are reflections of alternating periods of shell deposition and dissolution occurring during aerobic and anaerobic respiration, respectively. It might be speculated that for *Nautilus* the aerobic respiration occurs at night in warmer, shallower water, with anaerobic respiration during the day in colder, deeper waters. The issue of daily migratory habits that this latter thesis implies remains at present unresolved^{19,21,31}.

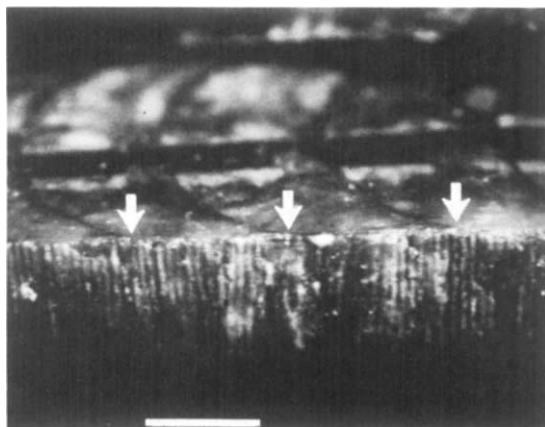
Other astronomical evidence

Other astronomical measurements of changes in the lengths of the day and the lunar distance may be compared to those obtained from our study of nautiloid growth rhythms. The short-term astronomical approach utilises careful measurements of the Moon's position through occultations timed by atomic clocks¹⁵. Although astrometry can determine precisely the rotation rate of the Earth, it cannot give the secular acceleration because, over periods from a few days to several centuries, the acceleration is dominated by non-cumulative fluctuations²⁶. Historical records of ancient solar eclipses¹⁶ provide a means of studying the evolution of the Earth–Moon system for the past 3,000 yr. This approach yields clear evidence that the lunar orbit and the length of the day have increased during this period. The lunar laser ranging experiment can now measure the distance to the Moon with an accuracy of about half of the predicted yearly lunar recession³⁵, and thus will soon be able to provide direct data on changes in the lunar distance.

From the ancient eclipse study¹⁶, the rate of change in the lunar orbit semi-major axis is $\dot{a}/a = 1.5 \times 10^{-10}$ parts yr^{-1} , or a recession of the Moon of 5.8 cm yr^{-1} . The value for the Earth–Moon distance 69.5 Myr ago is 0.840 times the present distance (Fig. 6). This implies an average change in the lunar orbit semi-major axis of 26.6×10^{-10} parts yr^{-1} or an average recession of 94.5 cm yr^{-1} . This is 17 times the rate from the ancient eclipse studies and implies (if this rate is the correct long-term one) that the recent dissipation is not typical for past geologic time and that additional energy sinks may be involved.

The unfortunate problem inherent in the astronomical approach is that there is no way of knowing whether the last few

Fig. 5 Dorso-ventral section of an orthocerid ~326 Myr old. The arrows mark the different chamber walls. There are a constant 15 ± 1 growth lines per chamber, the same number possessed by other nautiloid genera from almost time equivalent Mississippian formations. Scale bar, 1 cm.



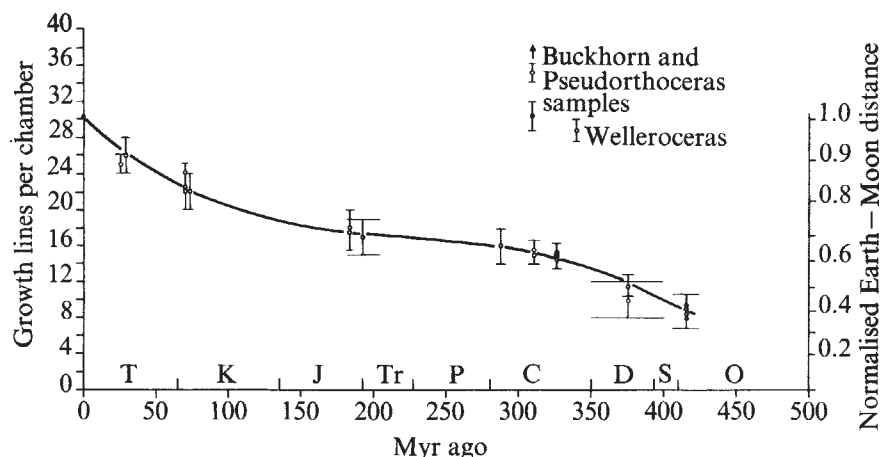
decades of lunar orbital evolution, or even the last few thousand years, are typical of the evolution which occurred over geological times. To extrapolate from a few thousand years to several hundred million is quite risky. Whether the rate of lunar orbital change for the last few thousand years is a 'minor fluctuation' compared to the millions of years of geologic time is not easily determined.

The only 'long-term' study (on a time scale of millions of years) is Wells' palaeontological study¹ on daily and annual banding in corals, the results of which suggest a much slower lunar recession than the nautiloid data does. Although the existence of annual banding on corals is well established, there is some question about daily banding in modern corals. We have seen no research indicating daily bands, except for Wells'

Table 1 Characteristics of available nautiloids

Years ago (millions)	Period	Location	Name	Specimen code	Chambers counted	Average growth lines per chamber	Day length (h)	Synodic month (h)	Sidereal month (h)	Earth-Moon distance (present distance = 1.0)	Standard deviation
0	Recent	New Caledonia	<i>Nautilus pompilius</i>	PK 1975A Cincin. Museum Natural History	5	29.4 ± 1					
0	Recent	New Caledonia	<i>Nautilus pompilius</i>	PK 1975B Cincin. Museum Natural History	7	29.0 ± 2					
0	Recent	S. Pacific	<i>Nautilus pompilius</i>	IA Princeton	4	30.5 ± 2					1.75
0	Recent	S. Pacific	<i>Nautilus pompilius</i>	IIB Princeton	2	33.5 ± 2	Average of 30.01 growth lines per chamber				Among recent specimens
0	Recent	S. Pacific	<i>Nautilus pompilius</i>	IIIC Colorado	6	29.75 ± 2	24.00	722	667	1.01	
0	Recent	S. Pacific	<i>Nautilus pompilius</i>	F Colorado	6	31 ± 2					
0	Recent	S. Pacific	<i>Nautilus pompilius</i>	G Colorado	8	30.6 ± 2					
0	Recent	S. Pacific	<i>Nautilus pompilius</i>	Bryn Mawr	3	30 ± 1					
0	Recent	S. Pacific	<i>Nautilus pompilius</i>	Princeton	2	30 ± 1					
25 ± 4	Miocene	Chipola Formation, Florida	<i>Aturia curvilineata</i>	Smithsonian 644819	5	25 ± 1	23.83	596	558	0.898	0.84
28.5 ± 2	Oligocene	'Olig.' from Australia	<i>Aturia</i> sp.	3262 Princeton	2	26 ± 2	23.80	619	578	0.919	2.83
69.5 ± 1	Upper Cretaceous	Pierre Shale, Wasta, S.D.	<i>Eutrophoceras dekayi</i>	D1652 USGS Denver	2	22 ± 2	23.52	517	488	0.882	2.83
69.5 ± 4	Upper Cretaceous	Pierre Shale, S. Dakota	<i>Eutrophoceras dekayi</i>	85219 Princeton	1	22.5 ± 2	23.52	529	499	0.833	0.48
69.5 ± 1	Upper Cretaceous	Pierre Shale, S. Dakota	<i>Eutrophoceras dekayi</i>	8688 Iowa	3	24 ± 1	23.52	564	530	0.868	1.00
69.5 ± 1	Upper Cretaceous	Pierre Shale, S. Dakota	<i>Eutrophoceras dekayi</i>	Smithsonian	2	22.5 ± 2	23.52	529	499	0.833	2.12
72.5 ± 2	Upper Cretaceous	Ripley Formation, Coon Creek, Tenn.	<i>Eutrophoceras dekayi</i>	Smithsonian	2	22 ± 2	23.50	517	488	0.821	2.83
183 ± 3	Lower Jurassic	Charmouth, Dorsetshire, England (LIAS)	<i>Nautilus inornatus</i>	W.4329 Princeton	3	18 ± 2	22.73	409	391	0.708	1.15
183 ± 3	Lower Jurassic	England	<i>Nautilus inornatus</i>	W.5147 Princeton	2	17.5 ± 2	22.73	398	380	0.696	0.71
192 ± 30	Lower Jurassic to Upper Triassic	England	<i>Cenoceras intermedius</i>	W.4331 Princeton	2	17.0 ± 2	22.67	385	369	0.681	0.71
287 ± 4	Upper Pennsylvanian	Graham Formation, Finis Shale	<i>Brachycycloceras normale</i>	SUI 609 Iowa	3	16.0 ± 2	22.00	352	339	0.644	0.48
314 ± 4	Middle Pennsylvanian	Laddsdale coal	<i>Metacoceras carinatum</i>	# 36306 Iowa	4	15.0 ± 1	21.82	327	316	0.614	0.82
314 ± 4	Pennsylvanian	Lower Cherokee Laddsdale coal	<i>Domatoceras</i> sp.	# 3610 Iowa	5	15.5 ± 1	21.82	338	326	0.627	0.55
314 ± 4	Pennsylvanian	Lower Cherokee Laddsdale coal	<i>Temnocheileus harneri</i>	# 36311 Iowa	4	15.0 ± 1	21.82	327	316	0.614	0.50
326 ± 2	Upper Mississippian	Imo Formation, Arkansas	<i>Reticycloceras peytonense</i>	SUI 36411 Iowa SUI 36409 Iowa	15	15.0 ± 1	21.74	326	314	0.613	0.26
326 ± 2	Upper Mississippian	Imo Formation, Arkansas	<i>Reticycloceras croneisi</i>	SUI 36397 Iowa W. B. Saunders	8	14.5 ± 0.5	21.74	315	304	0.599	1.26
326 ± 2	Upper Mississippian	Imo formation, Arkansas	<i>Reticycloceras imoense</i>	SUI 36331 Iowa W. B. Saunders	8	15.37 ± 0	21.74	334	322	0.622	0.00
326 ± 10	Upper Mississippian	Batesville Formation	<i>Orthoceras</i> sp.	A, B, C, D (4 specimens) Princeton	13 total	15.11 ± 1	21.74	328	317	0.615	0.35
375 ± 25	Lower-Mid Devonian	unknown	unknown	681 and W. 299 Princeton	2	10 ± 2	21.40	214	209	0.466	0.21
375 ± 3	Lower-Mid Devonian	Dublin, Ohio	<i>Orthoceras</i> sp.	U. of Iowa	7 total	11.5 ± 1.5	21.40	246	239	0.511	0.48
420 ± 10	Upper Ordovician	Maquoketa Formation, Iowa	<i>Charactoceras laddi</i>	U. of Iowa	1	8.0 ± 1	21.08	169	165	0.399	0.71
420 ± 10	Upper Ordovician	Oxford, Ohio	<i>Charactoceras baeri</i>	W.4319 A, B Princeton	5	9.0 ± 1	21.08	190	186	0.431	0.71
420 ± 10	Upper Ordovician	Kope Formation near Cincinnati	<i>Treptoceras</i> sp.	R. Davis Mus. Nat. Hist. Cincinnati	27	8.5 ± 0.5	21.08	179	176	0.415	0.50
420 ± 10	Upper Ordovician	Graf, Iowa	<i>Dolorthoceras sociale</i>	R. Davis Mus. Nat. Hist. Cincinnati	3	9.0 ± 1	21.08	190	186	0.431	1.85
420 ± 10	Upper Ordovician	Graf, Iowa	<i>Dolorthoceras sociale</i>	R. Davis Mus. Nat. Hist. Cincinnati	12	9.5 ± 0.5	21.08	200	196	0.447	0.58

Fig. 6 Plot of the number of growth lines per chamber length and Earth–Moon distance against geologic time showing the tendency towards increase in the number of nautiloid growth lines/chamber with time. The points are fitted by a third degree polynomial least squares fit. Note the consistency with which nautiloids of each era have the same number of growth lines per chamber, with the only exceptions being the three specimens of Carboniferous age, which reflect multiple integrals of the predicted constant number. Independent evidence suggests that these species, in contrast to the other nautiloids studied, lived in a shallow water regime subject to tidal interference. Vertical error bars mark the maximum discrepancy between any single growth line count and the average for the specimen. The horizontal width of each error bar reflects the uncertainty in the dating of each fossil. For changes in the Earth–Moon distance based on the nautiloid data (right scale), the steep slopes in the curve may represent higher tidal energy dissipation during the last 70 Myr as well as from 325–420 Myr ago. About 375 Myr ago the Moon was about one-half its present distance from the Earth.



observation of one coral species in which this seems to occur. Usually incomplete lunar monthly cycles reflect more the coral's environment of growth than lunar periodicity³⁶. Other researchers report that the maximum density of reef coral skeletons is only associated with periods of above average seawater temperature³⁷. Growth patterns vary considerably among modern individual corals, even of the same community³⁶. The potential of corals as geochronometers is seriously limited by the absence of daily and monthly growth patterns in vertical sections of coral skeletons³⁸.

The nautiloid data can provide long-term evidence for the length of the day. By assuming only that the angular momentum of the Earth–Moon system is conserved, the lengths of the day and month are tied inseparably to one another, and, therefore, are both established by the nautiloid data. The past length of the day has not been established by any other growth studies. Calculations on the length of day are being done and will reduce, when substituted into the Runcorn equation, the palaeo-lunar distances in Table 1 and Fig. 6.

Rate of lunar orbital evolution

A synthesis of palaeomagnetic data, stratigraphic relationships, and studies of environments of sediment deposition have led to a reconstruction of Mesozoic and Palaeozoic continental configurations³⁹, however, much more exact and extensive information on ocean profiles is required for even an order of magnitude determination of ancient tidal energy dissipation. Since the magnitude of tidal dissipation depends so strongly on ocean configurations and depths^{26,40}, theoretical extrapolation of the lunar orbit into the past becomes an impossible task⁴¹. Given this limitation, it is still a generally accepted characteristic of lunar orbital evolution that it proceeded most rapidly when the Earth and the Moon were closer together. Tidal forces were then greater because the tidal torque is proportional to $a^{-11/2}$ (ref. 14). This means that the orbit would have quadrupled in size in the first 10 Myr if the Moon originally had been near the Roche limit. However, when the present astronomically determined rate of the Moon's recession is extrapolated back in time, the Moon approaches the Roche limit less than 10^9 yr ago, according to Kaula¹³, and a lunar surface melt would have occurred due to tidal energy dissipation. There is no lunar or terrestrial geologic evidence to support such a close approach at that time, since the youngest rocks on the Moon were crystallised 3.25×10^9 yr ago. This represents a time scale problem which has not been satisfactorily explained.

The change in the Earth–Moon distance shown in Fig. 6 does not proceed at a constant rate, but exhibits rather several slopes, with the slope being most gentle when the continents were not

separated, 225 Myr ago. This may reflect less active mid-ocean ridges, a lower sea level, and thus a corresponding decrease in tidal friction, as well as a decrease in the rate of retreat of the Moon from the Earth^{42–44}.

The recession rate of the Moon during the last 420 Myr, based on nautiloid data, varies by a factor of less than 3, which does not support a recession rate varying by $a^{-11/2}$. This supports the explanation of the time scale problem in terms of changing palaeogeography. As Kaula¹³ points out, the formation of Pangea at a high latitude would lead to lower tidal dissipation. If tidal dissipation takes place mainly in shallow seas, then a lowering of sea level to a depth below the present day continental shelves could conceivably reduce the tidal dissipation⁴¹. However, the establishment of even non-tidal oceanic circulation patterns in the past is extremely difficult.

If the Moon originally was near the Roche limit about 4×10^9 yr ago, as Lowman⁴⁵ and others have proposed, then the orbit must have evolved slowly for the Moon to be at half of its present distance 375 Myr ago, in the Devonian, as our calculations show. This slower evolution can explain why the lunar maria are concentrated on the near side of the Moon, as well as why the Moon's centre of mass is displaced earthwards relative to its figure. If slower evolution occurred, the Moon may have been close enough to the Earth for gravitational accelerations asymmetric to the centre of the Moon to influence magma extrusion 3.5 to 4.0×10^9 yr ago⁴⁶. Certain aspects of the Witwatersrand sediments, deposited 2×10^9 yr ago, may be explained by larger tides due to a smaller Earth–Moon distance⁴⁷. Studies of Precambrian stromatolites^{48,49} suggested that greater palaeotides existed until 1.7×10^9 yr ago, a feature unexplainable if the Earth–Moon distance increased rapidly early in the system's history⁵⁰. It is difficult to extrapolate beyond the 420 Myr of nautiloid data without an understanding of why the recession does not vary inversely as the $11/2$ power of lunar distance. One must also explain why the recession rate observed in historical times is so much smaller than the long-term average shown by nautiloid data for the past 70 Myr.

A recent summary of geologic evidence on the accretion of continents⁵¹ provides new support for the idea of a lunar orbit slowly expanding from about 4×10^9 yr ago until the Cambrian. One or a few supercontinents may have accreted gradually beneath a globe-encircling ocean in the Precambrian, with the continent(s) emerging only 600 Myr ago, at the beginning of the Cambrian. We, therefore, support the existence of three stages in the dynamical evolution of the Earth–Moon system. First, at a time when liquid water was not present on the surface of the Earth, the lunar orbit evolved slowly due to tides in the solid Earth and Moon. Next, the Moon continued to recede slowly

because the deep sea encircling and covering the continents dissipated little energy. Then, about 600 Myr ago, when the continents emerged, the last stage began, and lunar recession proceeded more rapidly.

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DNA Sequence of the mini-insertion IS2-6 and its relation to the sequence of IS2

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In polar orientation IS2 abolishes galactose operon expression. Operon activity is restored by a 108 base pair mini-insertion within IS2 called IS2-6. The DNA sequences of the gal operon-IS2 junction, the parental IS2 region undergoing sequence rearrangements and IS2-6 itself are reported. IS2-6 is composed of sequence intervals present in both strands of IS2.

It is known that IS2 occurs in multiple copies in the *E. coli* chromosome¹ and to transpose to various sites in the same or other replicons, at which it sometimes induces mutations². The analysis of such mutations revealed that IS2 can influence the expression of adjacent genes in two different ways depending on its orientation of integration^{3,4}. For example, when integrated into the control region of the galactose operon of *E. coli* in orientation I, IS2 turns off the expression of the *gal* genes downstream in the direction of transcription; in the opposite orientation (orientation II) it efficiently turns them on. We have further shown that an IS2 element integrated in polar orientation (orientation I) can undergo internal changes that also lead to constitutive expression of neighbouring genes, although the gross orientation of the element remains unaltered^{3,5}.

To study the internal changes within IS2 more precisely, a recombinant plasmid, pDG1, was constructed, containing the galactose operon of *E. coli* K12 within the plasmid pSC101. IS2 was then transferred to plasmid pDG1 by homogenote formation, generating the mutation *galOP-308::IS2*. This new plasmid, harbouring IS2 in polar orientation in the control region of the galactose operon is called pDG12. A strain carrying a deletion in the *gal* region of the *E. coli* chromosome was transformed with pDG12 and used for further genetic manipulations⁵. At 37 °C Gal⁺ revertants of this strain are observed with a frequency of 3×10^{-7} . Among 109 such Gal⁺ revertants analysed 102 exhibited inducible synthesis of *gal* operon specific enzymes, indicating that the wild-type *gal* operon had been restored, most likely due to accurate excision of the integrated IS2 element. This has been verified for one such inducible revertant⁵. However, seven clones were found to be constitutive for *gal* enzyme synthesis, suggesting that other events had led to reactivation of the *gal* operon. The seven constitutive Gal⁺ revertants fall into three groups: Class 1 consists of one revertant, which was shown by genetic means to be due to a highly unstable suppressor located on the bacterial chromosome. Class 2 contains three revertants which show no gross alterations within IS2, as judged by analysis of DNA fragments produced by various restriction endonucleases. These revertants are stable.

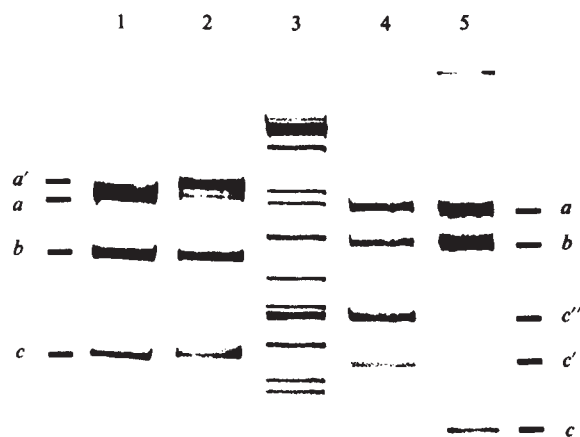


Fig. 1 Electrophoretic banding patterns of the *Hind*III DNA fragments of pDG12 (IS2) and pDG12 R1 (IS2-6), which are 895 and 1,010 base pairs long respectively⁵, after *Hinf*I and *Hae*III digestion. Slot 1, *Hinf*I digestion of pDG12 DNA, band *a*, *b*, and *c* correspond to 435, 282, and 153 base pairs respectively. Slot 2, digest of pDG12 R1 with *Hinf*I. Note the shift in molecular weight of band *a* (435 base pairs) to *a'* (542 base pairs). The presence of *a* material in pDG12 R1 is due to *Gal*⁺ segregants present in the DNA preparation. All other bands are unaffected by mutation R1. Slot 3, Marker λ dv-1 digested with restriction endonuclease *Hae*III. From bottom to top the molecular weights of the bands correspond to 120, 136, 150, 180, 185, 210, 240, (286, 331), 451, 481, 1,000, 1,325, and 1,460 base pairs respectively⁸. Slot 4, DNA fragments of pDG12 R1 produced by *Hae*III endonuclease. Bands *a*, *b*, *c'* and *c* correspond to 430, 340, 207, and 153 base pairs respectively. Slot 5, pDG12 *Hae*III fragments. Bands *a*, *b*, and *c* correspond to 430, 340 and 99 base pairs respectively. Plasmid DNA from pDG12 and pDG12 R1 was prepared as described previously⁵. Preparative isolation of *Hind*III fragments was performed using 6–8 mm thick agarose slab gels (1%); gel strips containing the bands were cut out and DNA was extracted^{9,10}. DNA was pelleted by adding 0.3 M Na-acetate was 2.5 volumes of ethanol. For terminal labelling and further enzymatic cleavage this DNA was purified by one cycle ethidium bromide–CsCl buoyant density gradient centrifugation. Digestion of the DNA (0.5–1.0 μ g) with restriction enzymes *Hinf*I and *Hae*III was performed in 10 mM Tris–HCl buffer pH 7.6, 10 mM MgCl₂, 1 mM DTT, 50 mM NaCl, and 0.1 mM EDTA, and 100 μ g ml⁻¹ carboxymethylated BSA. The digest was adjusted to 10 mM EDTA and 10% glycerol, 0.05% bromophenol blue was added, and the mixture was subjected to electrophoresis on 10% polyacrylamide slab gels in 40 mM Tris-acetate buffer pH 8.3 for 2–3 h at 110 V. The gels were stained in water containing 2 μ g ml⁻¹ ethidium bromide for 10–15 min. The gel was then photographed under ultraviolet light.

Class 3 consists of three further revertants which are characterised as being highly unstable. These were previously shown to be due to the addition of small DNA segments within IS2. Revertant R1 contains an insertion of 115 base pairs, while in R3 a 65-base pair DNA addition was observed. These mini-insertions were previously termed IS6 and IS7 respectively⁵.

We report here the DNA sequence of part of IS2 and of IS6. These results indicate that IS6 can be considered an allele of IS2. Therefore it will henceforth be called IS2-6. The DNA sequence of IS2 and IS2-6 reported here suggests a model for the formation of mini-insertions.

Mapping of IS2-6

In an earlier report the presence of additional DNA within IS2 in two unstable constitutive *Gal*⁺ revertants of *galOP*-308::IS2 on pDG12 was shown by agarose gel electrophoresis of DNA fragments produced by restriction enzymes⁵. The relevant DNA fragment used in that study was produced by restriction endonuclease *Hind*III.

While the *Hind*III fragment generated from plasmid pDG12 is 895 base pairs long, the corresponding fragment produced from DNA presumably carrying IS2-6 (pDG12 R1) is 1,010 base pairs long. When the *Hind*III fragment from pDG12 is further digested with *Hae*III endonuclease, three smaller frag-

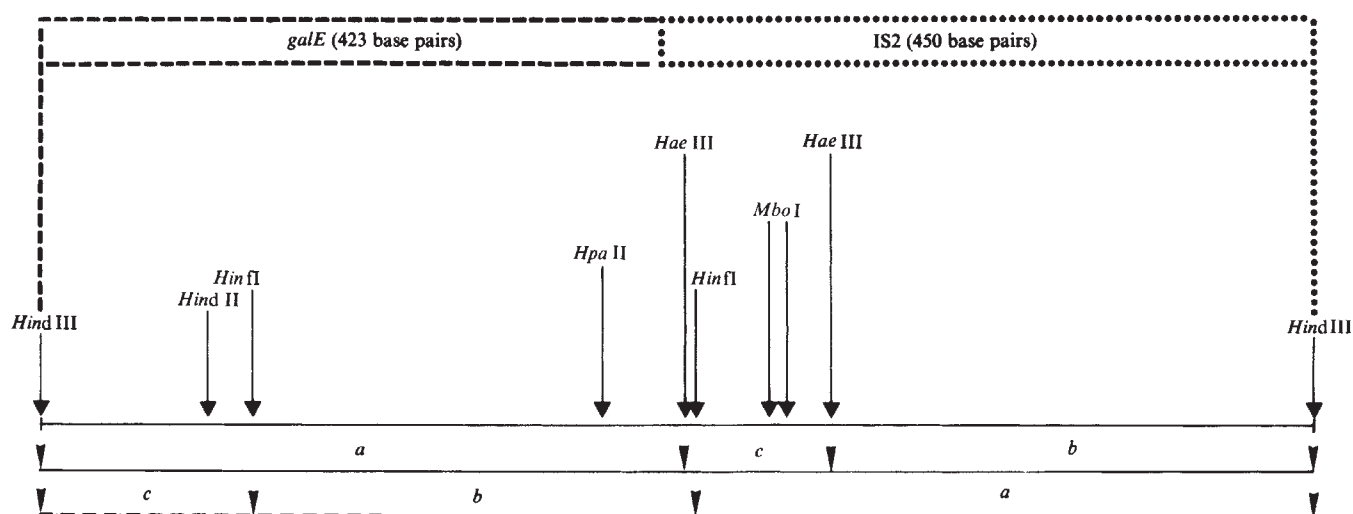
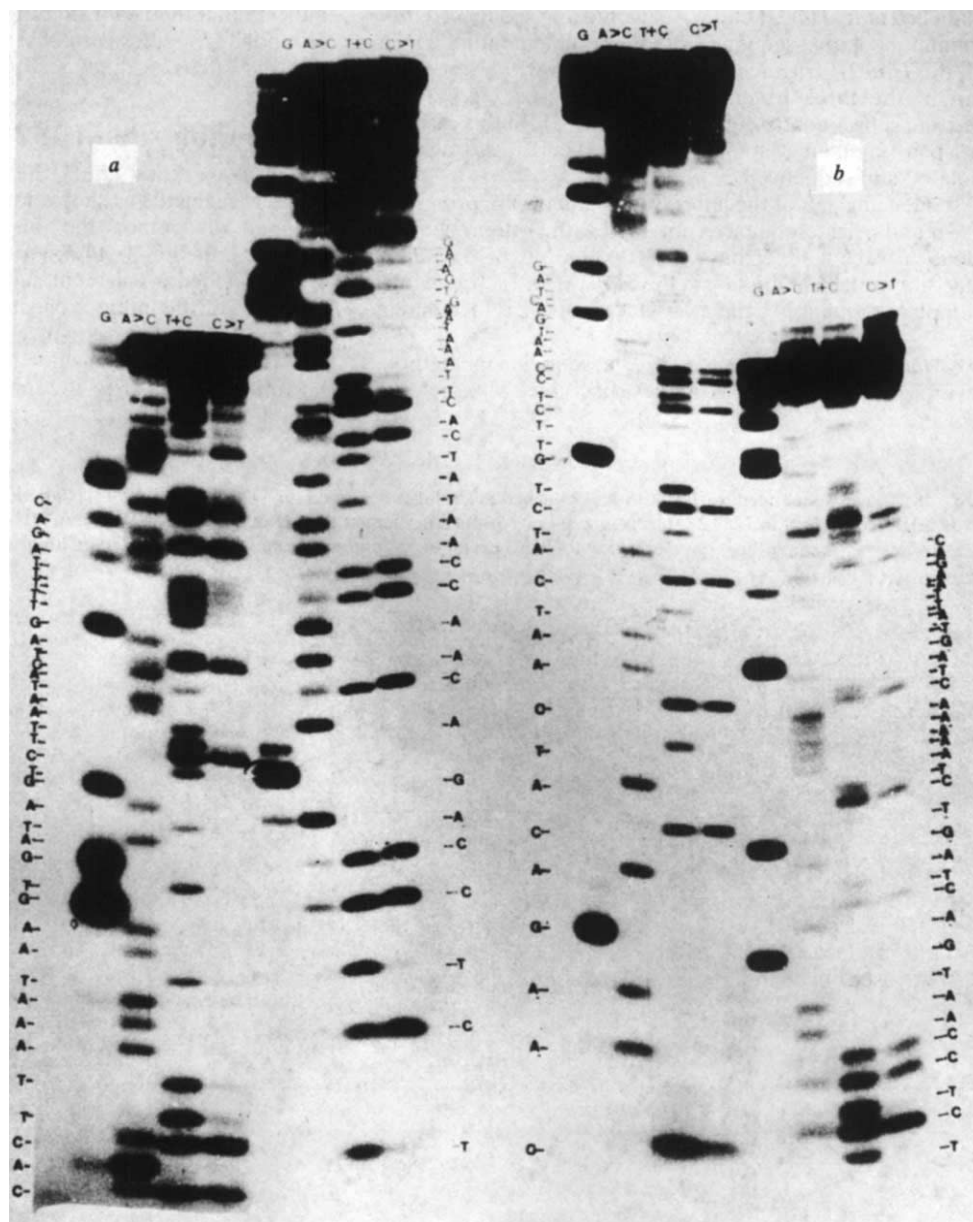


Fig. 2 Detailed restriction map of the 895-base pair⁵ *Hind*III DNA fragment of pDG12. The pertinent sites for cleavage by restriction endonucleases *Hind*II, *Hae*III, and *Hinf*I are shown, including one *Hpa*II site. The *Mbo*I sites are inferred from the sequencing data, but the enzyme does not cleave at these positions (see text). The map was established by digesting the *Hind*III fragment with one enzyme and comparing this with the reciprocal double digest of combinations of various other enzymes. The *Hinf*I and the *Hae*III fragments *a*, *b*, and *c* are given below the map. When terminally labelled *Hind*III fragment of pDG12 is digested with *Hae*III (see Fig. 1, slot 5) fragments *a* and *b* contain the label at one of their 5'-ends, while fragment *c* is not labelled. This shows that fragment *c* is derived from the interior part of the *Hind*III fragment. The position of fragment *a* can readily be identified since it contains the known *Hind*II cleavage site within *galE* (ref. 5). *Hinf*I digestion of the *Hind*III fragment also yields three fragments *a*, *b*, and *c* (see Fig. 1, slot 1). Digestion of *Hae*III fragment *a* with *Hinf*I yields a 153- and a 275-base pair fragment. Digestion of terminally labelled *Hae*III fragment *c* with *Hinf*I generates a 7- and a 92-base pair fragment. This shows that one *Hinf*I cleavage site is located in *galE*, while the other is close to a *Hae*III site within IS2. The only *Hpa*II site shown is generated after digestion of the 282-base pair long *Hinf*I fragment *b* and thus maps at a position already described^{11,12}. The total length of the *Hind*III fragment as determined by the sum of the proper fragments is 873 instead of 895 base pairs as determined previously⁵.

Fig. 3 Autoradiograms of sequencing gels. *a*, [32 P]*Hinf*I/*Hae*III fragment from nucleotide 18–74 of IS2. *b*, [32 P]*Hae*III/*Hinf*I fragment from 106–52 of IS2. Both sequences read from the labelled 5'-end and represent complementary DNA strands. The sequences overlap at 53–74. About 10–20 pmol of *Hae*III or *Hinf*I fragments of either IS2 or IS2-6 were dephosphorylated with 0.5–1 unit of bacterial alkaline phosphatase (Worthington, phenolysed, ether extracted and ethanol precipitated). Fragments were labelled using [γ - 32 P]ATP (1,000–3,000 mCi mmol $^{-1}$, NEN) and 2 units of polynucleotide kinase (Boehringer Mannheim). The doubly labelled fragments were separated on a polyacrylamide gel and the corresponding bands were eluted. These were redigested with either *Hinf*I or *Hae*III to yield a fragment labelled at only one 5'-end. DNA sequencing was performed by partial methylation (for G), controlled partial hydroxynolysis (for C and T) and strong alkaline treatment at 90 °C (for A > C) followed by strand scission as described in ref. 6. Products were analysed in 15 or 20% denaturing polyacrylamide gels using Tris-borate-EDTA buffer followed by X-ray autoradiography.



ments (*a*, *b*, and *c*) are generated (Fig. 1, slot 5). However, digestion of the 1,010 base pair long fragment of pDG12 R1 with restriction endonuclease *Hae*III yields three major (*a*, *b*, and *c'*, slot 4) and two minor DNA fragments (*c* and *c'*, slot 4). Fragment *c*, not visible in Fig. 1, is identical in size and sequence to the 99 base pair long fragment *c* of pDG12. It is generated by molecules which have lost the mini-insertion within IS2. Frequent spontaneous loss is characteristic of IS2-6. The amount of fragment *c* found in pDG12 R1 preparations correlates with the number of Gal $^{-}$ segregants among cells harbouring plasmid pDG12 R1 (ref. 5).

Fragment *c'* is 153 base pairs long. It is a deletion derivative of IS2-6 and accumulates in cultures with time. Its isolation, properties, and DNA sequence will be reported elsewhere (Ghosal and Saedler, manuscript in preparation).

Two of the three major fragments (*a* and *b*) of the parental and the Gal $^{+}$ constitutive revertant plasmid DNA pDG12 R1, are identical in size. Fragment *c* of pDG12 is 99 base pairs long while the corresponding fragment of pDG12 R1, fragment *c'*, is 207 base pairs long. The mini-insertion IS2-6 is therefore 108 base pairs long and has arisen by insertion within the small

*Hae*III fragment *c* of pDG12, yielding fragment *c''* of pDG12 R1. Both fragments, *c* and *c''*, (Fig. 1) have been used for DNA sequence analysis.

The location of *Hae*III fragment *c* within IS2 and with respect to the most proximal *gal* structural gene is shown on the restriction map of the 895-base pair long *Hind*III fragment of pDG12 presented in Fig. 2.

DNA sequence of part of IS2

The DNA sequence between the *Hpa*II site and the rightmost *Hae*III cleavage site on pDG12 (Fig. 2) was determined using the procedure described by Maxam and Gilbert⁶.

In one set of experiments the *Hind*III fragment of pDG12 carrying *galOP*-308::IS2 was isolated and digested with *Hae*III endonuclease. The mixture of three smaller fragments which results was dephosphorylated and the 5'-ends were labelled with [γ - 32 P]ATP using polynucleotide kinase. Fragment *c* (see Fig. 1) was isolated and its labelled strands separated by gel electrophoresis. These were subsequently sequenced.

In an alternative procedure, the labelled *Hae*III fragment *c* was further digested with *Hinf*I. The larger 92-base pair frag-

ment labelled at its *Hae*III end was isolated and sequenced. For determination of the sequence of the complementary DNA strand, the *Hind*III fragment was first cleaved with *Hinf*I. The mixture of the three fragments generated (see Fig. 1) was labelled and subsequently digested with *Hae*III. In this case the 92-base pair fragment is labelled at its *Hinf*I end. This fragment was isolated and sequenced.

The sequencing gels of the latter two fragments are presented in Fig. 3a and b. The sequences obtained with both procedures are identical except for 7 base pairs to the left of the *Hinf*I cleavage site, which are missing in the *Hinf*I-*Hae*III fragments. The complete sequence of the *Hae*III fragment c is included in Fig. 5.

Two *Mbo*I sites are detected by the sequencing method. However, *Mbo*I does not cleave at these positions. This seems to

indicate that the *Mbo*I sites are modified in this bacterial background. This agrees with observations by others that the *Mbo*I cleavage site is modified in *E. coli* K12.

Integration site of IS2

On cleavage with *Hinf*I the *Hind*III fragment yields three smaller fragments as shown in Fig. 1, slot 1. The 282-base pair fragment b contains the junction between IS2 and the *gal* operon. Labelling both 5'-ends and subsequent digestion with *Hpa*II yielded a fragment suitable for sequencing. The DNA sequence of the integration site of IS2, which also contains the *gal* mRNA leader sequence and the total sequence of the *Hae*III fragment c is included in Fig. 5. It can be seen that IS2 has integrated next to the initiation site for *gal* mRNA tran-

Fig. 4 IS2-6 was sequenced by the methods described in the legend to Fig. 3. *a*, DNA sequence of the labelled IS2-6 *Hinf*I fragment *a'* cleaved with *Hae*III, from position 36-128. *b*, DNA sequence of the complementary DNA strand of IS2-6 from 201-116 (labelled *Hae*III fragment *c'*, cleaved with *Hinf*I). The overlapping region is thus 13 base pairs long.



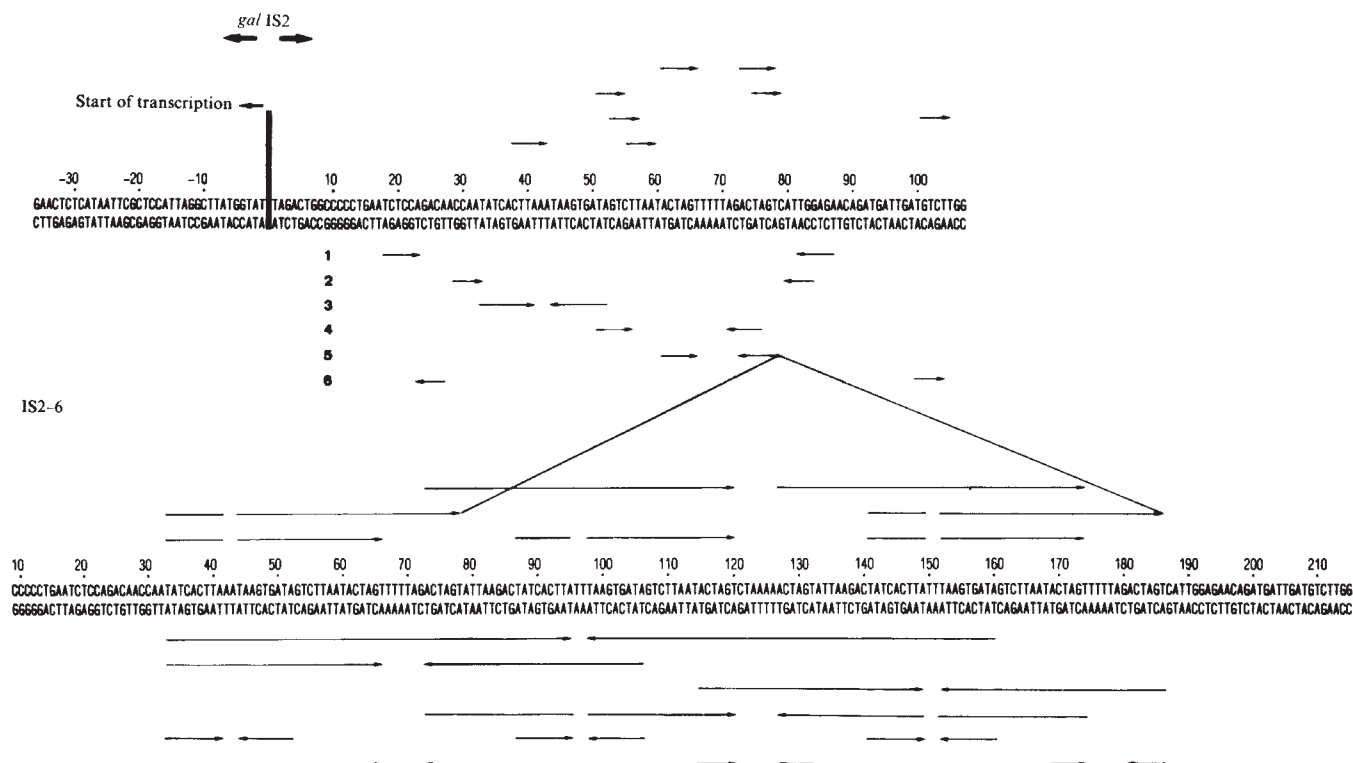


Fig. 5 Sequences with negative numbers represent material from the galactose operon, others are IS2 and IS2-6 sequences, respectively. The vertical bar in the top line separates the *gal* from the IS2 sequences. The arrows above the sequences indicate direct sequence repetitions, while the arrows below the sequences show the inverted repeats. For IS2 the small inverted repeats are numbered from top to bottom. The connecting lines between IS2 and IS2-6 show the length and the position of the 108-base pair insert of IS2-6.

scription. Thus in mutation *galOP*-308, IS2 disconnects the mRNA initiation site from the *gal* promoter. Comparison with the sequence of the operator proximal end of IS2 reported for *galOP*-490::IS2 (ref. 7) reveals that in both strains IS2 has integrated into the control region of the *gal* operon, but at two different sites, separated by one nucleotide. In addition the comparison also reveals that the termini of IS2 correspond to imperfect inverted repeats.

Features of the IS2 sequence

Figure 5 also shows that there are a few small direct and inverted repeats in the sequence of the *Hae*III fragment *c* of IS2. Six pairs of inverted repeats are present. It should be noted that the sequence between the left end of pair 3 and the right end of pair 5, that is, nucleotide pair 33 to nucleotide pair 78, is especially rich in A-T (76%), while the A-T content of the flanking sequences is about 50%.

Features of the sequence of IS2-6

The same strategy has been used to analyse IS2-6, the *Hae*III fragment containing IS2-6, being 207 instead of 99 base pairs long.

The sequencing gels for the *Hinf*I/*Hae*III fragments are given in Fig. 4a and b. The DNA sequence obtained is included in Fig. 5. Except for the modified *Mbo*I sites already seen in the IS2 sequence, no other new cleavage sites for restriction endonucleases are found in IS2-6.

The most striking feature of the IS2-6 sequence is the high degree of symmetry. The large inverted repeat is 62 base pairs long and is substructured as indicated. In addition there is large perfect duplication of 46 base pairs in length as well as five smaller imperfect direct repeats. The A-T content between positions 33 and 186 is 76%.

Comparison of IS2 and IS2-6

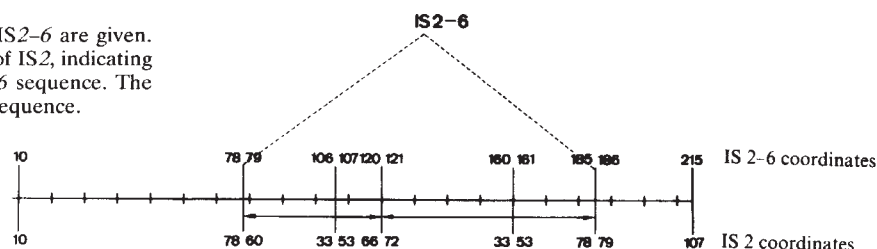
Figure 5 shows that IS2-6 constitutes an insertion of 108 base pairs within IS2 at either position 78/79 or position 44/45. Assuming position 78/79 to be the point of DNA addition, it is clear that the 108 base pairs of IS2-6 are derived from sequences of IS2 located between positions 33 and 78, that is, from IS2 material contained between the inverted repeat pairs 3 and 5.

However, IS2-6 does not consist of a simple duplication of IS2 sequences from these regions. Instead sequences of both IS2 strands located within the 33-78 interval are multiplied and rearranged in the manner outlined in Fig. 6. Some IS2 sequences are represented several times in both possible orientations in IS2-6. The 53-60 region of IS2, for example, recurs five times, while the pairs of inverted repeats 3 and 5 are found three times and in both orientations.

Mode of formation of IS2-6

One explanation of how the complicated structure outlined in Fig. 6 might have arisen is that recombinational events occurred between the two DNA strands of IS2 during replication.

Fig. 6 The coordinates of one DNA strand of IS2-6 are given. The numbers below the line are the coordinates of IS2, indicating which sequences of IS2 are present in the IS2-6 sequence. The arrows emphasise the orientation of the sequence.



However, the high degree of symmetry observed in IS2-6 suggests another mechanism of formation based exclusively on replication. This mode of formation will be described briefly (Fig. 7) as it enables predictions to be made about the sequences of other replication products that would be expected to occur.

Considering the replication of the lower DNA strand of IS2 only, the DNA synthesising enzyme may be imagined to proceed from the 5'-end across the inverted repeat at position 33/52 (pair number 3) to position 78 (Fig. 7a). Because of the high A-T content of this region, partial melting might occasionally

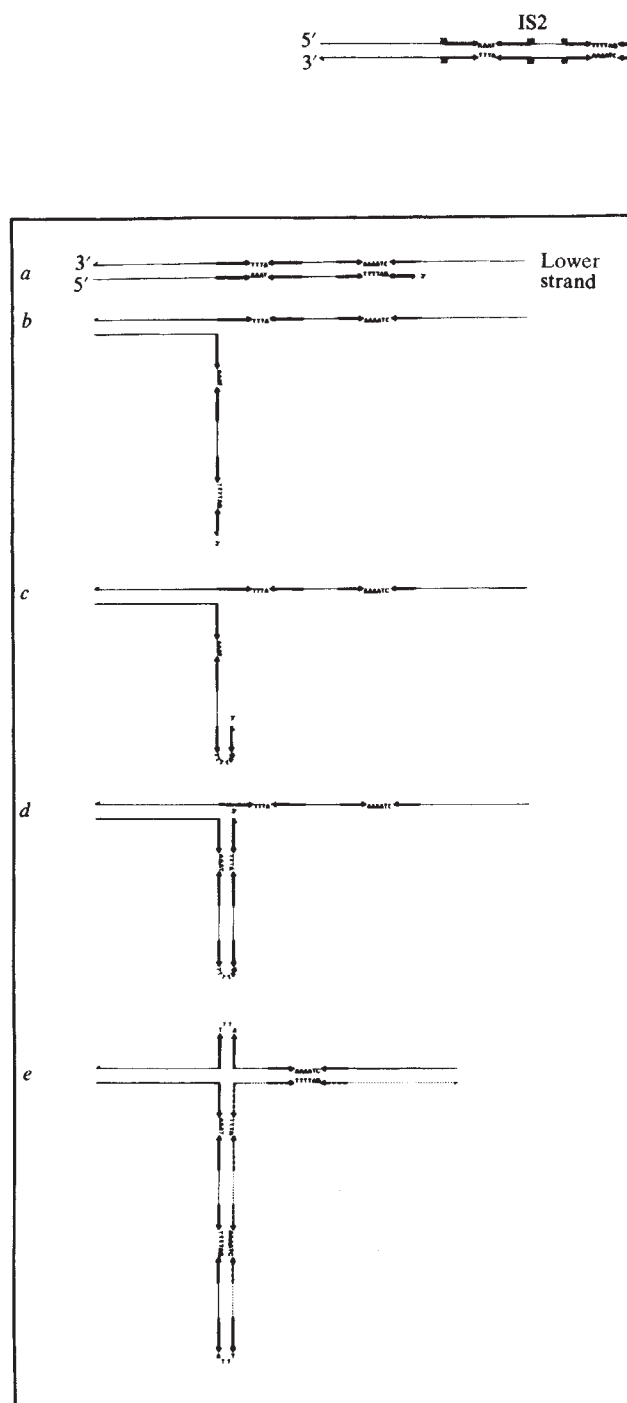
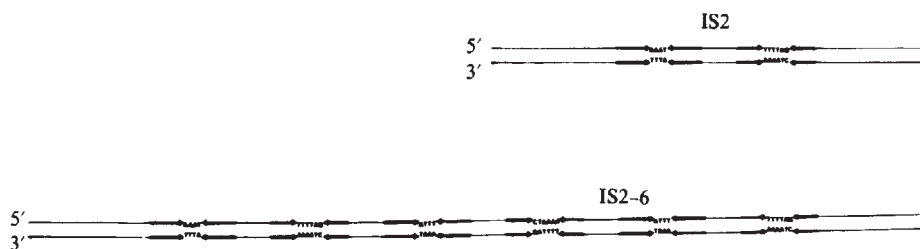


Fig. 7 Slippage replication of the lower DNA strand of IS2 (upper frame) indicate the positions of the inverted repeat pairs 3 and 5 (see Fig. 5), which are marked by heavy arrows throughout. The hypothetical sequence of events (a-e) leading to the replication products IS2 and IS2-6 are enclosed by a black line. For simplicity only the DNA sequences separating both parts of an inverted repeat pair are shown. e, dotted and dashed lines indicate DNA synthesised after a second round of slippage of the DNA template. The segregation products of the heteroduplex formed are presented in the lower frame as IS2 and IS2-6. See text for details.



occur (Fig. 7b). Subsequently inverted repeat pair 5 of the newly synthesised DNA strand could fold back on itself forming a new template for further DNA synthesis (Fig. 7c). The polymerase could then proceed in the 3' direction along the newly synthesised strand until it passes inverted repeat pair 3 and reaches position 33 (Fig. 7d). At this point the same slippage process is repeated: melting out, folding back of the inverted repeat pair 3 and DNA synthesis along the newly made DNA strand leading to the structure shown in Fig. 7e. This slippage tends to form long inverted repeats. Occasionally these may resist partial melting out so that DNA synthesis can then proceed along the old parental DNA strand, provided that inverted repeat pair 3 (33–52) in the parental DNA strand has folded up as indicated in Fig. 7e to yield a stable structure.

On another round of replication the DNA heteroduplex molecule forms two segregants, the old parental IS2 sequence and the newly generated IS2–6 allele.

The slippage of the DNA template during replication of IS2 as outlined in Fig. 7 generates symmetrical DNA additions (see Fig. 5). The process requires, first, a DNA region rich in A–T, and second, pairs of inverted repeat sequences at both ends of the A–T rich region. The newly formed sequences generated by this process may contain new signals for the turn-on of gene expression, as shown by IS2–6. Which parts of the IS2–6 sequences constitute the turn-on signal is not yet known.

The replication 'slippage' model described in Fig. 7 for IS2–6 allows us to predict the DNA sequence of the replication 'slippage' product of the upper DNA strand of IS2. Since preparation of this manuscript we have determined the sequence of the 54-base pair mini-insertion IS2–7. Its sequence is identical to the slippage product predicted for replication of the upper strand of IS2 and will be reported elsewhere¹³.

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Phenotypic expression in *E. coli* of a DNA sequence coding for mouse dihydrofolate reductase

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The construction and analysis of bacterial plasmids that contain and phenotypically express a mammalian genetic sequence are described. Such plasmids specify a protein that has enzymatic properties, immunological reactivity and molecular size characteristic of the mouse dihydrofolate reductase, and render host cells resistant to the anti-metabolic drug trimethoprim.

SINCE the initial propagation of eukaryotic DNA in bacteria¹, several systems have been used to study the expression in *Escherichia coli* of DNA derived from higher organisms. Biological activity of genes from the lower eukaryotes, *Saccharomyces cerevisiae*^{2,3} and *Neurospora crassa*⁴, has been demonstrated using phenotypic selection for functions that complement mutationally inactivated homologous bacterial genes. Immunological reactivity with antibody made against human somatostatin was shown for a peptide fragment cleaved *in vitro* from a hybrid protein encoded in part by bacterial DNA and in part by a chemically synthesised somatostatin DNA

sequence⁵. Very recently, a protein containing amino acids of rat proinsulin was shown to be made by bacteria that carry a double-stranded complementary DNA (cDNA) transcript of pre-proinsulin mRNA⁶; in that instance, antigenic determinants for both insulin and the bacterial enzyme β -lactamase were detected on a fused peptide transported outside the cell. It is not known, however, whether the mammalian peptide components of such immunologically reactive hybrid proteins have functional biological activity.

Our approach to the study of mammalian gene expression in bacteria has been to generate a heterogeneous population of clones carrying a DNA sequence that codes for a selectable mammalian gene product, and then to select directly those bacteria in the population that phenotypically express the genetic sequence. The mammalian enzyme dihydrofolate reductase (DHFR), which catalyses the conversion of dihydrofolic acid to tetrahydrofolic acid, is especially suitable for this purpose. The mammalian DHFR has a much lower affinity for the antimetabolic drug, trimethoprim (Tp), than does the corresponding bacterial enzyme⁷. Thus, bacteria which biologically express mammalian DHFR activity are resistant to levels of trimethoprim that ordinarily inhibit growth.

When these studies were initiated, the only bacterial host approved for EK2 recombinant DNA experiments⁸ was *E. coli* K12 strain χ 1776 (ref. 9). As this strain is already resistant to

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high concentrations of trimethoprim because of its *thy*⁻ mutation¹⁰, direct selection of bacteria that synthesise the mammalian DHFR could not be carried out. Therefore, our initial studies used an *in situ* hybridisation procedure¹¹ to identify χ 1776 clones which carried a mouse DHFR cDNA sequence, with the expectation that a highly sensitive indirect radioimmunoassay¹² would be used to detect any clones that expressed antigenic determinants of the protein product encoded by the gene.

Construction and cloning of chimaeric plasmids containing a DHFR ds cDNA

Figure 1 summarises the experimental scheme used in this study. Partially purified mRNA containing DHFR sequences from methotrexate-resistant AT-3000 mouse cells¹³ was used in the preparation of double-strand (ds) cDNA by RNA-dependent DNA polymerase (reverse transcriptase) and DNA polymerase I (ref. 14 and Fig. 2). Homopolymeric deoxy-C 'tails' were added to the unfractionated cDNA by terminal deoxynucleotidyl transferase (Fig. 2) and the section of the gel containing the predominant (1,500 base pairs) tailed cDNA was eluted. The material recovered from the gel was annealed with an equimolar concentration of pBR322 plasmid DNA that had been cleaved in the β -lactamase gene by the *Pst*I endonuclease¹⁵ and treated with terminal transferase to add homopolymeric dG tails at the cleavage sites. The extent of annealing of DHFR ds cDNA with the plasmid vector was monitored by electron microscopy¹⁶ using circle formation as an indicator. *Pst*I sites are regenerated at both ends of the insert as a result of such recircularisation^{17,18}.

Constructed chimaeric plasmids were introduced into MnCl₂-treated¹⁹ *E. coli* K12 strain χ 1776 and tetracycline (Tc)-resistant transformants (yield ~30 colonies per ng DNA) were selected and tested separately for the presence of a DNA species complementary to a highly purified DHFR cDNA probe. About 40% of the Tc-resistant clones gave a positive reaction by an *in situ* hybridisation test¹¹ (Table 1 and Fig. 3). Plasmids from 14 of the reacting clones were examined by gel electrophoresis and all were found to contain a single DNA insert approximately 1,500 base pairs in length. As both ends of the *Pst*I-cleaved pBR322 DNA receive homopolymeric dG tails, only those molecules that have acquired a dE-tailed cDNA insert or had escaped cleavage and/or dG tailing would be expected to re-circularise and transform; thus, we presume that most of the nonreacting

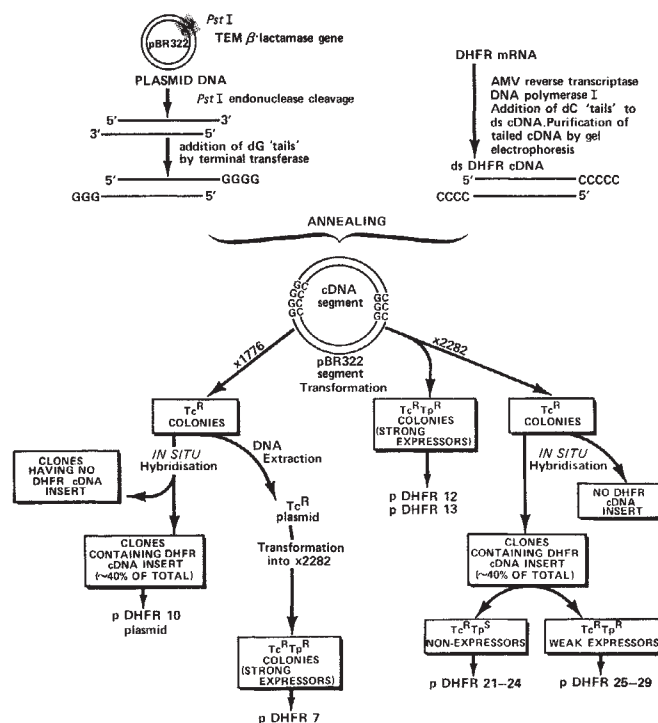


Fig. 1 Scheme used for cloning and expression of mouse DNA sequences that code for DHFR. Additional experimental details are given in the legend to Table 1 and in the text.

Tc-resistant colonies contain inserts of contaminating non-DHFR cDNA.

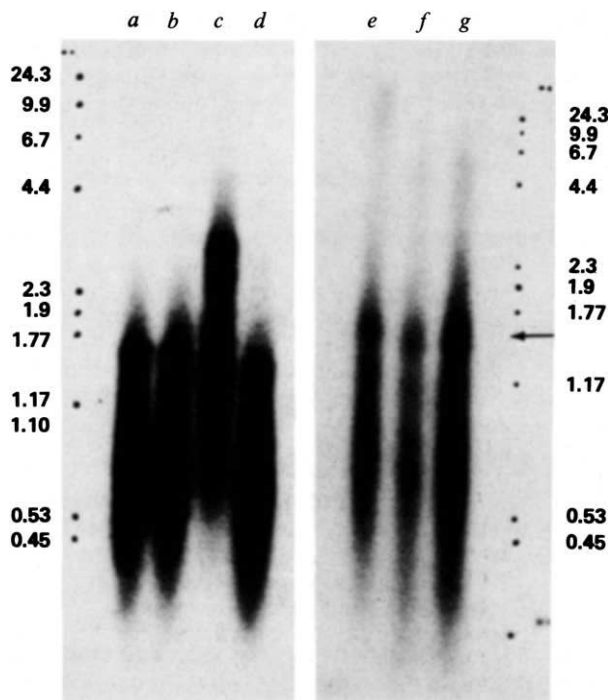
When χ 2282, a *thy*⁺ variant of χ 1776, was approved for EK2 use, direct selection of bacteria that phenotypically expressed the eukaryotic DNA sequence became feasible (see Fig. 1). Two populations (25 colonies each) of previously identified Tc-resistant colonies of χ 1776 were pooled, and plasmid DNA extracted from these populations was introduced by transformation into χ 2282. In other experiments, we transformed χ 2282 directly with annealed pBR322-DHFR cDNA (Table 1). Colonies that expressed resistance to both Tc and Tp were obtained in both types of experiments; three independent

Table 1 Transformation experiments using χ 1776 and χ 2282

Host	Transforming DNA		Transformants per ng DNA			<i>In situ</i> hybridisation with DHFR cDNA probe (% positive)
	Plasmid vector	Insert	Tc (5 μ g ml ⁻¹)	Tc (10 μ g ml ⁻¹)	Tc/Tp (5 μ g ml ⁻¹ of each)	
χ 1776	pBR322	None	4×10^2	2×10^2	$< 2 \times 10^{-3}$	—
χ 1776	pBR322	cDNA(1°)	60	32	—	40
χ 2282	pBR322	cDNA(1°)	70	—	2	44
χ 2282	pBR322	cDNA(2°)	60	—	1.3×10^{-1}	—
χ 2282	pDHFR7	—	75	—	25	—

pBR322 plasmid DNA that had been annealed *in vitro* with dC-tailed DHFR cDNA (designated 1°) was introduced into χ 1776 or χ 2282, using a modification of a previously described transfection procedure¹⁹. 1 ml of an overnight bacterial culture was inoculated into 100 ml of L broth supplemented with diaminopimelic acid (DAP, 50 μ g ml⁻¹) and (for χ 1776 only) thymidine (4 μ g ml⁻¹). Bacterial cultures were grown until exponential phase at 35°C and then collected by centrifugation at 4°C. Cells were washed in 0.3 volume 10 mM NaCl, resuspended in 30 ml freshly prepared MCN buffer (70 mM MgCl₂; 40 mM sodium acetate, pH 5.6, and 30 mM calcium chloride) and chilled on ice for 20 min. Cells were collected, resuspended in 1 ml MCN and added in 200- μ l aliquots to 50 μ l DNA in TEN (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 50 mM NaCl) or MCN buffer. After chilling at 0°C for 30 min, reactions were incubated at 27°C for 5 min, chilled again for 30 min, and 50- μ l samples were plated on to Penassay broth agar supplemented with DAP, thymidine (for χ 1776), and antibiotics as indicated. When χ 2282 was used, the selective medium was M9 minimal agar supplemented with 0.5% casamino acids, biotin (2 μ g ml⁻¹), DAP (50 μ g ml⁻¹) and Tp (2.5–10 μ g ml⁻¹) plus tetracycline (Yc) or kanamycin (Km) as indicated. Plates containing transformants were incubated at 32°C and colonies were scored 2–3 d after plating. pBR322 plasmid DNA lacking the cDNA insert was used as a control. cDNA preparations labelled as 2° consisted of plasmid DNA isolated from a nonfractionated population of clones that had previously been transformed with chimaeric molecules carrying a cDNA insert. In the experiment shown in the last line the transforming DNA was isolated from a clone (pDHFR7) that expresses resistance to Tp as well as Tc.

Fig. 2 Preparation and characterisation of DHFR cDNA insert. DNA complementary to DHFR mRNA was synthesised essentially as described elsewhere¹⁴, using avian myeloblastosis virus (AMV) reverse transcriptase and polysomal RNA obtained by indirect immunoprecipitation of DHFR-synthesising polysomes from methotrexate-resistant AT-3000 S-180 mouse cells¹³. The RNA had been estimated to contain DHFR mRNA as 20% of its mRNA¹³. The reaction was carried out in 100 μ l using 340 μ g polysomal RNA (estimated to contain 5 μ g polyA-RNA), 45 units AMV reverse transcriptase and dCTP labelled to 4 Ci mmol⁻¹ with ³²P-dCTP (Amersham). Approximately 1.4 μ g cDNA was synthesised in 30 min. The reaction was stopped by the addition of EDTA (to 10 mM) and extracted with phenol, followed by ether, before being passed over a Sephadex G-50 fine column in 10 mM Tris, pH 7.4, 2 mM EDTA and, 10 mM NaCl (TEN). The void volume was collected and precipitated with ethanol. After centrifugation, the RNA was hydrolysed with NaOH (ref. 31), neutralised and precipitated with ethanol. The cDNA was then used as template for the synthesis of the second strand by *E. coli* DNA polymerase I essentially as described³¹. The reaction took place at 42 °C for 10 min in 100 μ l using 1.1 μ g cDNA, 10 units DNA polymerase I, and 200 μ M of each deoxynucleotide triphosphate with dCTP adjusted to 30 Ci mmol⁻¹ as above. Approximately 0.85 μ g of the second strand was synthesised. The reaction was stopped and extracted as above before being passed over a Sephadex G-50 fine column in TEN containing only 0.1 mM EDTA. Column fractions containing the ds cDNA were then treated with *Aspergillus oryzae* S₁ nuclease as described elsewhere³¹. After extraction and precipitation with ethanol, approximately 1.0 μ g ds cDNA was obtained. Aliquots of *a*, first strand product; *b*, first strand product after base treatment; *c*, second strand product and *d*, second strand product after S₁ nuclease treatment were examined on a 1.5% agarose gel in alkaline conditions as described³². Terminal addition of dCTP to the ds cDNA by terminal deoxynucleotidyl transferase (TdT), prepared as described elsewhere³³ was carried out by a modification³⁴ of the Co²⁺ procedure³⁵. The reaction was carried out in 500 μ l containing 140 mM cacodylic acid, 30 mM Tris base, 110 mM KOH (final pH 7.6), 0.1 mM dithiothreitol, 150 μ M dCTP (adjusted to 8 Ci mmol⁻¹ with ³H-dCTP (Amersham)), 1 mM CoCl₂ (added to prewarmed reaction mix before enzyme addition), approximately 1.0 μ g ds cDNA (assuming an average MW giving approximately 600 base pairs, this provides 10 pM 3' termini per ml) and 0.5 μ l TdT (2.3 $\times$ 10⁵ units ml⁻¹). The reaction was allowed to proceed at 37 °C for 10 min before being cooled and sampled to determine incorporation. Approximately 30 dC residues were added per 3' terminus. The reaction was stopped, extracted, desalted and precipitated with ethanol as above. Aliquots of *e*, second strand product; *f*, second strand product after S₁ nuclease treatment, and *g*, dC-tailed ds cDNA were analysed on a 1.7% agarose gel in Tris-acetate-NaCl (ref. 36). The dC-tailed ds cDNA was then preparatively electrophoresed on a similar gel and the '1,500-base pair' region (arrow) cut out of the gel and electrophoretically eluted into a dialysis bag as described elsewhere³⁷. The eluted material was extracted as above, concentrated by lyophilisation and precipitated with ethanol. After centrifugation, the 1,500-base pair dC-tailed ds cDNA (approximately 80 ng) was redissolved in 10 mM Tris HCl, pH 7.4, 0.25 mM EDTA and 100 mM NaCl (annealing buffer). pBR322 plasmid DNA, isolated as described elsewhere³⁸ was digested with a 1.5-fold excess of *Pst*I endonuclease in conditions suggested by the vendor (New England Biolabs) and the linear plasmid DNA was cut out and eluted from a 0.7% agarose gel in TBE³⁹ as described above. The plasmid DNA was 'tailed' with dG residues using procedures similar to those described above. Approximately 15–20 dG residues were added per 3' terminus. Following extraction, the dG-tailed vector was passed over a Sephadex G-50 fine column in annealing buffer and the void volume was collected. Equimolar amounts of dC-tailed ds cDNA and dG-tailed vector DNA were allowed to anneal essentially as described by W. Rowe and R. A. Fratell (personal communication) except that the vector concentration was kept at 75 ng ml⁻¹ in the annealing reaction. Circularisation was monitored by electron microscopy¹⁶ and was typically about 20–40%. This annealed DNA was used directly for transformation into χ 1776 or χ 2282.



transformants were found to be capable of immediate growth in media containing at least 1,000 μ g ml⁻¹ of Tp, and were termed 'strong expressors'. Plasmid DNA isolated from these colonies was shown by repeat transformation to encode both the Tc and Tp resistance phenotypes. However, Tp^RTc^R transformants occurred in different experiments at only 20–60% of the frequency observed when selection of transformants was carried out for Tc resistance alone. Transfer of Tc-selected colonies on to minimal medium agar plates containing Tp showed that all such clones express resistance to at least 1,000 μ g ml⁻¹. Together, these findings suggest that phenotypic expression of Tp resistance may be delayed in transformants until a sufficient quantity of plasmid-specified DHFR has accumulated. Analogous results have been obtained with other antimicrobial drug-resistance determinants encoded by plasmids introduced into *E. coli* by transformation²⁰.

Structure of the DHFR cDNA

Gel electrophoresis of endonuclease-cleaved plasmid DNA from three separately derived bacterial clones that expressed high levels of trimethoprim resistance showed similar overall patterns. Using such data, a cleavage map (Fig. 4) of the cDNA insert of one of these (pDHFR7) was constructed.

Examination of the amino acid sequence of the mouse DHFR enzyme²¹ allowed us to assign one of the *Hae*III cleavage sites to the *trp-pro* (TGG-CCX) present at amino acid positions 24 and 25, thus localising the DHFR structural sequence to a position near the 5' end of the mRNA template used in synthesising the cloned cDNA. Other cleavage sites within the DHFR structural sequence were consistent with positions predicted by computer analysis of the amino acid sequence. DNA sequence analysis of two separate regions of the cDNA provided direct verification that the nucleotide sequence of the insert corresponds to the amino acid sequence reported for the mouse DHFR enzyme, and also confirmed that the coding sequence for DHFR is located at the ds cDNA equivalent of the 5' end of the mRNA.

The nucleotide sequence at the pBR322-cDNA junction nearest the 5' end of the mRNA used as template for DHFR cDNA (that is, at the *Pst*I_a site) is of special interest. The complement of the 'sense' strand of β -lactamase gene of the vector (J. G. Sutcliffe, personal communication) is interrupted at the *Pst*I site by a series of 11 dG residues added by the terminal transferase, and these are followed immediately by (1) an ATG (AUG) protein start codon, and (2) the codon for the first amino acid of the mouse DHFR structural gene. The sequence that codes for the mouse DHFR is in the same orientation as at that encoding the β -lactamase on the vector plasmid; however, the

number of incremental G residues (that is, 10) at the vector-insert junction ensures that the DHFR cDNA sequence is not in the same translational reading frame as the β -lactamase gene.

If the phenotypic expression we have observed for the mouse DHFR sequences in bacteria is the result of translational readthrough from signals that initiate protein chains within the β -lactamase gene, then the host bacterial cell must be able to circumvent the observed frame shift by a 'slippage' mechanism of translation. Such an event might potentially be aided by the long run of dG residues introduced at the pBR322-cDNA junction and could account for our observation (Table 1) that plasmids containing a DHFR cDNA insert yield more than twice as many expressors as would be expected from considerations of reading frame and orientation. However, the high level of functional expression observed for both primary and secondarily transformed clones of pDHFR7 does not seem to be readily explained by slippage of tRNA molecules during translation.

Perhaps a more likely explanation is that the *Pst*I-polyG-ATG sequence that has been constructed preceding the coding sequence for DHFR serves as a binding and protein initiation site for the bacterial ribosome. Recent studies^{22,23} have identified sequences on mRNA in the 5' direction from the initiator codon that are complementary to the CCUCC sequence at the 3' end of the 16S ribosomal RNA species, proposed by Shine and Dalgarno²⁴ to be involved in the binding of mRNA to ribosomes. It is tempting to speculate that the mRNA transcript from the sequence at the pBR322-cDNA

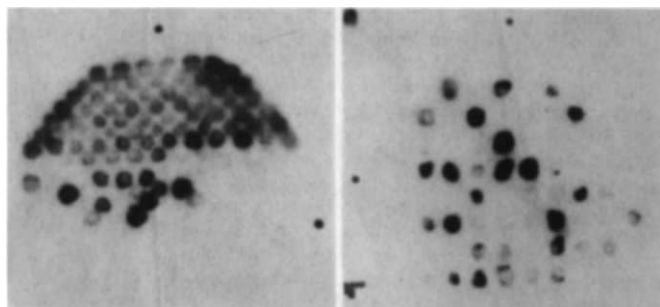


Fig. 3 Detection of colonies containing DHFR cDNA inserts by *in situ* hybridisation. Colonies were screened for DHFR sequences using a modification (G. N. Duell, unpublished) of an *in situ* hybridisation procedure¹¹. Tc-resistant colonies were transferred to nitrocellulose filters (Millipore, HAWG) that had been placed on Penassay broth agar plates containing Tc (10 μ g per ml). (Filters had been washed twice by boiling in H₂O and autoclaved before being placed on plates.) After 2–3 d of bacterial growth at 32°C, the filter was removed from the plate and placed on a Whatman no. 3 pad saturated with 0.5 M NaOH. After 7 min, the filter was sequentially transferred to a series of similar pads saturated with 1 M Tris, pH 7.4 (twice, 7 min each); 1.5 M NaCl, 0.5 M Tris, pH 7.5 (once, 7 min); and 0.30 M NaCl, 0.03 M Na citrate (2 $\times$ SSC) (once, 7 min). After the excess liquid had been removed by suction, the filter was placed on a pad containing 90% ethanol, dried by suction and baked *in vacuo* at 80°C for 2 h. Before hybridisation, filters were pretreated for 3–6 h at 65°C in hybridisation buffer that contained 5 $\times$ SSC, pH 6.1 0.2% SDS, 0.02% Ficoll 400 (Pharmacia) and 8 μ g ml⁻¹ *E. coli* tRNA. Hybridisations were carried out with individual filters in 1.5 ml hybridisation buffer containing 2 $\times$ 10⁴ c.p.m. ³²P-labelled purified DHFR cDNA¹³ in a sealed plastic bag at 65°C for 24 h. The filters were then washed in hybridisation buffer (once, 60 min at 65°C); in 5 $\times$ SSC, pH 6.1 (three times, 60 min each at 65°C); and in 2 $\times$ SSC, pH 7.4 (twice, 10 min at room temperature), air dried, and prepared for autoradiography. Left: top, a collection of χ 1776 colonies which contain a DHFR cDNA insert; middle, Tc-resistant χ 2282 colonies derived from transformation with annealed pBR322 ds cDNA—both reacting and non-reacting colonies are seen; bottom, colonies containing pBR322 and pACYC101 plasmids which show no visible hybridisation. Right: several positive colonies on a representative filter analysed in screening χ 1176 transformants. Negative colonies represent clones containing pBR322 or pACYC101 plasmids which show no visible hybridisation.

junction has sufficient complementarity to the CCUCC sequence to allow ribosomal binding when a translational start signal is located an appropriate distance away. In such an event, the ATG protein start signal that immediately precedes the coding sequence for the mouse DHFR might initiate a peptide chain having a size characteristic of the mammalian enzyme. Immunological analysis of extracts derived from pDHFR7 and other expressing clones has yielded results consistent with this interpretation (see below).

Analysis of enzyme activity encoded by pDHFR7 plasmid

Mammalian dihydrofolate reductases can be distinguished from their bacterial counterparts by the ability of mammalian enzymes to use folate as a substrate and by their differential sensitivity to competitive inhibitors^{25,26}. In initial experiments, the reduction of folate to tetrahydrofolate was measured using extracts from the pDHFR7 clone, from a Tp-sensitive clone containing a DHFR cDNA insert (the pDHFR10 plasmid), and from cells that contain only the pBR322 vector. Although all three clones are capable of synthesising a chromosomally produced bacterial enzyme, only the enzyme present in extracts from cells containing the pDHFR7 plasmid gave reduction of folate (4 $\times$ background). Additional evidence that the reductase encoded by the pDHFR7 plasmid is of mammalian origin was obtained by inhibitor analysis (Fig. 5). The DHFR isolated directly from mouse cells and the activity encoded by the pDHFR7 plasmid showed identical sensitivities to methotrexate, trimethoprim and a triazine derivative (2,4,-diamino-1-(4'-butylphenol)-6,6 dimethyl-1, 6-dihydro-1,3,5-triazine); both enzyme activities were 200 times more sensitive to the triazine than to trimethoprim. In contrast, bacterial dihydrofolate reductase is inhibited more effectively by trimethoprim ($K_i = 5 \times 10^{-9}$) than it is by triazine ($K_i = 6.5 \times 10^{-4}$)²⁵.

As methotrexate binds stoichiometrically to dihydrofolate reductase²⁷, we can estimate the number of molecules of enzyme in the pDHFR7 plasmid extracts from the methotrexate inhibition data of Fig. 5. We calculate from the specific activity of the extract (3 units per mg of soluble protein) and the specific activity and methotrexate binding parameters of the mouse enzyme²⁸ that 0.01% of the soluble bacterial protein is active mammalian DHFR.

Immunological characterisation of bacterial cell extracts containing mouse DHFR

Immunological evidence confirming the nature of the DHFR encoded by pDHFR7 and other plasmids that contain a mouse DHFR cDNA insert was obtained using a solid-phase sandwich radioimmunoassay¹². Tp^r clones of χ 2282 containing the independently derived plasmids pDHFR 7, 12 and 13 showed a strong reaction with rabbit antibody directed against mouse DHFR in an *in situ* immunoassay¹² (data not shown); protein that reacted with the antibody was also made by bacteria which showed low levels of phenotypic expression and by some clones that did not make a biologically functional DHFR (that is, were Tp sensitive). The nature of the antigen synthesised by Tp^s and Tp^r clones was examined more fully using a newly developed method (filter affinity transfer, or FAT procedure) for the *in situ* immunological characterisation of proteins in gels²⁹. This procedure depends on the covalent coupling of F(ab)₂ antibody fragments to a chemically derivatised and activated cellulose filter; antigen transferred on to the filter from an SDS-polyacrylamide gel is detected by subsequent incubations with antiserum and ¹²⁵I-labelled *Staphylococcus aureus* protein A (ref. 12).

Filter affinity transfer analysis of the pDHFR7 extract (Fig. 6, lanes b, c) shows the presence of protein that reacts immunologically with the antibody to mouse DHFR and further shows

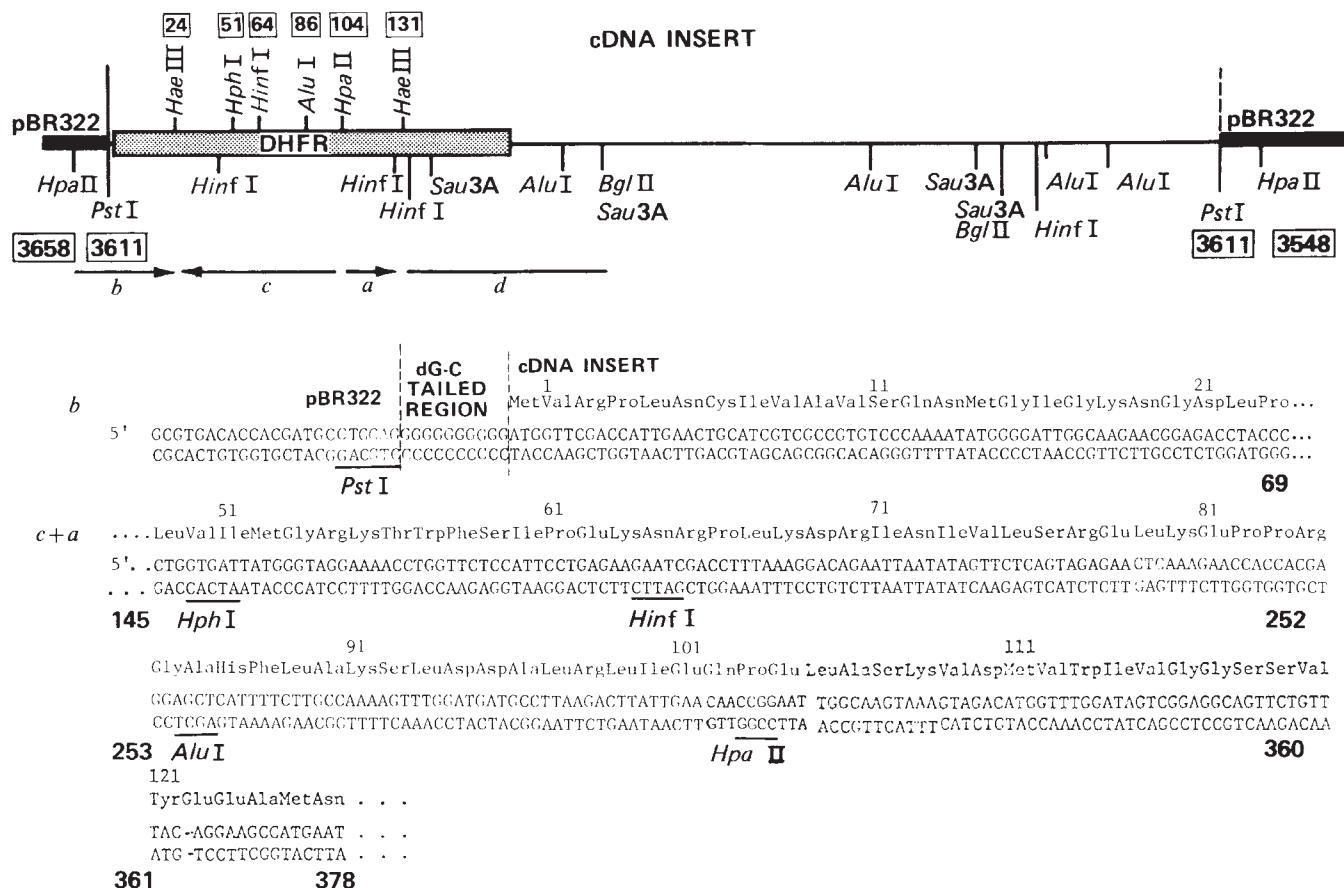


Fig. 4 Map of cDNA insert and adjacent regions of pDHFR 7 plasmid. Endonuclease cleavage map and partial DNA sequence of cDNA insert of the pDHFR 7 plasmid. The shaded area indicates the structural sequence for mouse DHFR. The locations of endonuclease cleavage sites were determined from polyacrylamide gel electrophoresis patterns following simultaneous or sequential digestions of either intact pDHFR or fragments isolated after earlier digestions. Restriction endonucleases (New England Biolabs or Bethesda Research Laboratories) were used according to the vendor's recommendations. Cleavage sites listed above the shaded area were assigned to specific amino acid positions within the mouse enzyme by nucleotide sequence. The boxed numbers of the *HpaII* and *PstI* sites indicate the locations of the sites in the pBR322 plasmid as determined by Sutcliffe (personal communication), and were used in orientating the amino acid sequence of the DHFR gene with respect to the β -lactamase sequence. The nucleotide sequence in the vicinity of the pBR322–cDNA junction corresponding to the 5' end of the mRNA used as template for the cDNA, and the sequence in the region of the *HpaII* site at amino acid 104 of the structural sequence are shown. Nucleotides and amino acids are numbered from the start of the DHFR coding sequence; nucleotides in the 5' direction on the mRNA from position 1 have negative numbers. The DNA sequence shown were determined by the method of Maxam and Gilbert⁴⁰. Fragments (*d* + *c*) and (*a* + *b*) were 5' end-labelled at the *HpaII* and *BglII*-generated ends, treated with *HaeIII* endonuclease, and subjected to electrophoresis in 6% acrylamide gel and TBE buffer³⁹. Fragments *a*, *b* and *c* were eluted from the gel³², precipitated with ethanol and used directly for base sequence determination using the gel system described in refs 40 and 41.

that most of the immunologically reactive material has the same electrophoretic mobility as enzyme obtained directly from mouse cells (molecular weight 22,000) (Fig. 6, lane *a*). An immunologically reactive band that migrates at this position was also seen in the material eluted with folic acid from a methotrexate affinity column that contained an extract from χ 2282 (pDHFR7) cells (Fig. 6, lane *d*), suggesting that the 22,000 MW protein made by these bacterial cells has binding sites for both methotrexate and folate. Additional immunologically reactive bands which have mobilities consistent with a MW of 30,000–90,000 were seen in varying amounts in different extracts of bacterial cells that contained pDHFR7 (Fig. 6, lanes *b*, *c*); as the 1,500-base pair insert in this plasmid is capable of coding for a polypeptide no larger than 50,000 MW, we conclude that the most slowly moving bands are likely to be hybrid proteins that include antigenic sites of the mouse DHFR. Immunologically reactive high MW proteins are also made by a Tp^s clone (pDHFR21, Fig. 6, lane *e*) which contains a DHFR cDNA insert; however, this clone fails to synthesise an immunologically reactive 22,000 MW band. No immunological reactivity with

antibody made to mouse DHFR was detected in extracts of cells carrying only the pBR322 vector (Fig. 6, lane *f*).

Variation in level of expression of mouse DHFR cDNA sequences in bacteria

Thirty-two separately derived clones of χ 2282 transformants that had been selected on plates containing only Tc were replated on medium containing both Tc and Tp, and also were tested for the presence of a DHFR cDNA insert by *in situ* hybridisation. Fourteen colonies contained sequences homologous with the purified DHFR cDNA probe, and five of these (termed 'weak expressors') grew on plates containing 5 μ g ml⁻¹ or more Tp. The remaining nine clones that contained DHFR sequences failed to show any growth on concentrations of Tp above 2.5 μ g ml⁻¹, and were termed 'non-expressors'. Plasmid DNA isolated from four of the nine non-expressors and from all five weak expressors was analysed by gel electrophoresis, and the mean inhibitory concentration (MIC) of Tp was determined for each of the clones. The structural relationship of the cDNA

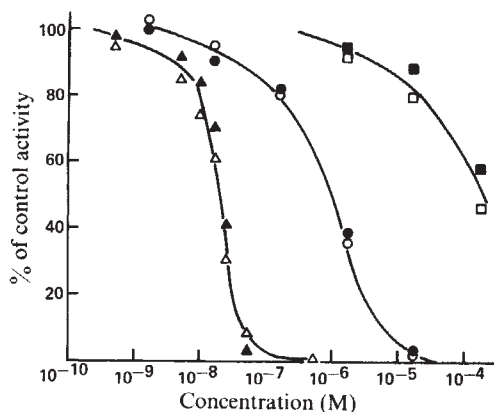


Fig. 5 Inhibitor analysis of DHFR from bacterial cells. Stationary phase cultures of χ 2282 expressing trimethoprim resistance were grown in the presence of Tp ($1 \mu\text{g ml}^{-1}$) in minimal medium, washed with isotonic saline, and suspended in 50 mM potassium phosphate buffer, pH 7.0, containing 10 mM benzamidine and 10 mM phenyl methyl sulphonyl fluoride (3 volumes buffer to 1 volume cells). The suspension was sonicated and centrifuged at 10,000 r.p.m. for 15 min. The supernatant was centrifuged for 1 h at 100,000g before being studied. An R_2 methotrexate-resistant mouse cell extract was prepared as described elsewhere⁴². Enzyme activity was measured by the radioactive folic acid assay previously described²⁸. Protein was determined by the method of Lowry⁴³. Approximately 3 units of activity from the χ 2282 extract or 5 units from the methotrexate-resistant mouse cell extract were incubated with inhibitor for 10 min at 24°C before assaying for folate reductase activity; the concentrations shown represent the final concentration of inhibitor in the reaction mixture. Background values, determined by measuring enzyme activity in the presence of 10 mM methotrexate, have been subtracted from all points. The results presented are the average of duplicate samples which generally varied by less than 10% and are expressed as a percentage of the value obtained in the absence of inhibitor. One unit of activity is the amount of enzyme needed to reduce 1 nmol of folate in 15 min at 37°C. Δ and $\blacktriangle$ indicate addition of methotrexate, $\circ$ and $\bullet$ indicate addition of the triazine derivative, and $\square$ and $\blacksquare$ indicate trimethoprim addition for χ 2282 and the mouse cell extracts, respectively.

insert to the β -lactamase gene sequence of the vector, the length of each insert, and the minimal inhibitory concentration (MIC) determined for the clone are shown in Fig. 7. As can be seen, plasmids pDHFR7, 12, 13 and 26–29 all contain a complete DHFR structural sequence inserted in the same orientation (that is, orientation a) as the gene encoding the bacterial β -lactamase. The clone carrying each of these plasmids expresses Tp resistance, although the MIC varies from $150 \mu\text{g ml}^{-1}$ for pDHFR28 to $>1,000 \mu\text{g ml}^{-1}$ for pDHFR7, 12 and 13; the greatest reactivity with antibody to mouse DHFR occurs with pDHFR12 (unpublished data).

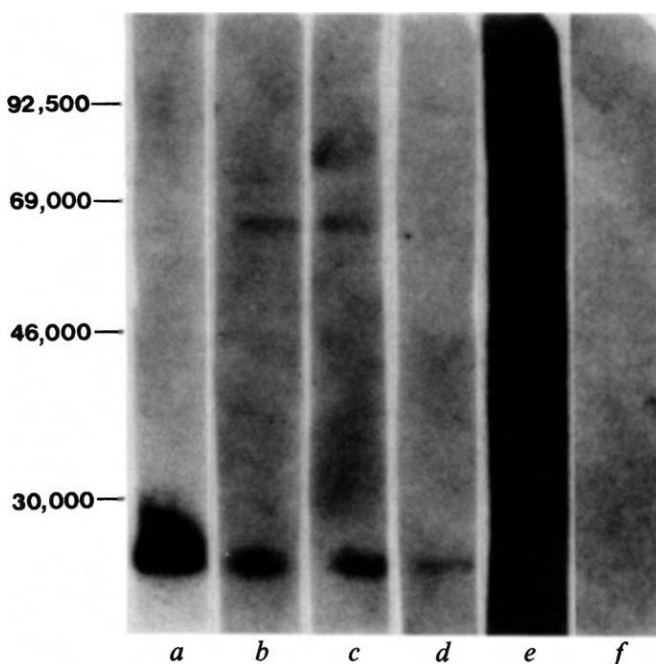
It is unlikely that translational reading frame is the determining factor in the different levels of expression observed in these clones, as our DNA sequence analysis indicates that a correct reading frame is not essential for efficient expression in pDHFR 7, 12 or 13. However, the positioning of the putative ribosomal binding site in relation to the ATG start codon may potentially influence the strength of expression by affecting the formation of the translational initiation complex (compare with ref. 23) (Figs 4, 7).

The end of DHFR structural sequence that corresponds to the 5' end of the mRNA is not present in plasmids pDHFR 21 and 24, thus explaining the observed lack of functional expression of the cDNA in these clones. However, pDHFR21, which has lost less than 15% of the structural sequence, nevertheless encodes a (probably hybrid) peptide that contains antigenic sites which

react with antibody to mouse DHFR (Fig. 6 and unpublished data). It is particularly interesting that the clone carrying pDHFR25 expresses a low level of Tp resistance, although the coding sequence for the DHFR enzyme is inserted in an orientation opposite to that of the β -lactamase gene. This finding, and our detection in extracts of the pDHFR25 clone of protein that reacts immunologically with antibody to the mouse enzyme (unpublished data), suggest that readthrough transcription into the DHFR coding sequence from a promoter sequence located on or near the distal segment of the β -lactamase gene may occur. Consistent with this interpretation are preliminary data suggesting that DHFR antigenic sites are also synthesised by cells carrying pDHFR23, which is a non-expressor of Tp resistance and contains a cDNA insert in orientation b (Fig. 7). Further study is required to determine whether sequences in the distal segment of the β -lactamase gene are capable of serving as weak promoters for the initiation of mRNA chains that extend into the DHFR cDNA.

The findings reported here indicate that the bacterial clones we have constructed are synthesising and phenotypically expressing DHFR encoded by mouse cDNA sequences: (1) the cDNA insert cloned in bacteria has been shown by *in situ* hybridisation to be homologous with the mouse gene and by direct DNA sequence analysis to encode the amino acid sequence of mouse DHFR, (2) DHFR enzymatic activity and resistance to Tp are specified by nucleotide sequences present on chimaeric plasmids but not on the vector, (3) the DHFR

Fig. 6 Filter affinity transfer analysis²⁹ of bacterial cell extracts. 20 μl of extracts in SDS sample buffer were run at constant current for 3 h in an 11.25% SDS-polyacrylamide slab gel. The gel was incubated in PBS (50 mM phosphate buffer containing 0.15 M NaCl) for 30 min and placed on a blotter wet with PBS. Peptides were specifically transferred from the gel to strips of a dry cellulose filter that had been covalently coupled to anti-DHFR F(ab)₂ fragments¹². Filters were washed, incubated with antibody to DHFR, washed again and treated with ^{125}I -labelled protein A. After additional washing and drying steps, the filters were analysed by autoradiography. The eluate fraction (lane d) was obtained by passage of an extract of χ 2282 cells containing pDHFR7 over a 0.5 ml methotrexate-Sepharose affinity column. The extract was acidified to pH 5.8, passed over the column and the bound fraction was eluted with 2 mM folic acid in a 5 mM NaHCO_3 buffer at pH 8.5 as described elsewhere²⁸. Lane a, extracts of mouse cell line; lanes b and c, pDHFR 7; lane d, eluate from methotrexate column; lane e, pDHFR21; lane f, pBR322.



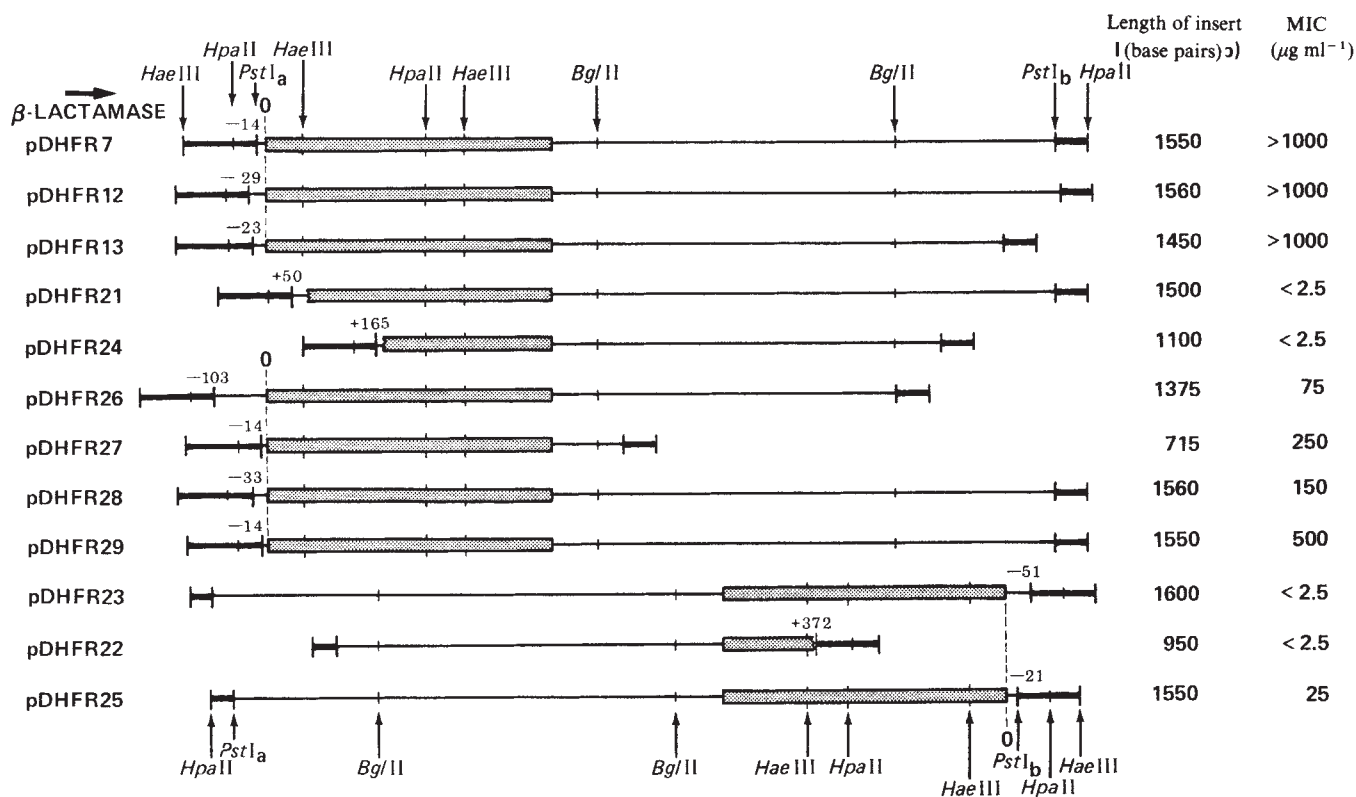


Fig. 7. Some structural and functional properties of chimaeric plasmids and χ 2282 clones containing DHFR cDNA insert. The MIC of Tp for each of the χ clones was tested on M9 minimal agar plates containing biotin ($2 \mu\text{g ml}^{-1}$), casamino acids (0.5%), DAP ($50 \mu\text{g ml}^{-1}$) and Tp concentrations ranging from 0 to $1,000 \mu\text{g ml}^{-1}$. χ 2282 (pBR322) was used as a control and is sensitive to Tp at $2.5 \mu\text{g ml}^{-1}$, as determined by incubation at 32°C for 3 d. Plasmid DNA isolated from each clone was digested with *Pst*I endonuclease¹⁵ at 37°C for 3 h and extracted sequentially with phenol and ether. DNA was precipitated with ethanol, resuspended in $8 \mu\text{l}$ TE buffer and electrophoresed in 1.2% agarose gels in TBE buffer to determine the length of the inserted fragment. ColEI plasmid DNA digested with *Hae*II endonuclease⁴² was added to each sample as an internal molecular weight standard. Additionally, *Hind*III-generated fragments of SV40 DNA were used as an external standard⁴⁵. The standard error is ± 100 base pairs with the method used. The orientation of the cDNA inserts in the vector plasmid was determined by gel analysis of plasmid DNA digested with *Bgl*II and *Hinc*II endonucleases; in addition, pDHFR25 was treated with the *Eco*RI and *Bgl*II enzymes to confirm the orientation of its insert. The direction of transcription of the β -lactamase gene of pBR322 is indicated by an arrow. The shaded area in each plasmid map indicates the structural sequence for DHFR. The numbers shown above each *Pst*I_a site indicate the distance (in base pairs) between the cleavage point and the first nucleotide of the DHFR structural sequence as described in Fig. 4. This distance was determined exactly by DNA sequence analysis for plasmids pDHFR 7, 12 and 13 and was estimated for the other plasmids by gel electrophoresis of fragments produced by either the *Hae*III or *Hpa*II endonucleases. For the chimaeric plasmids that we subsequently sequenced, this estimate proved to be accurate within 5 base pairs. *Pst*I–*Bgl*II distances were determined for the chimaeric plasmids by gel electrophoresis using *Hpa*II-cleaved pBR322 DNA as an internal standard. Nucleotides in the 5' direction on the mRNA from position 1 have negative numbers and are estimates obtained from gel analysis for all plasmids except pDHFR7 and 12.

encoded by the constructed plasmids shows differential sensitivity to competitive inhibitors of DHFR characteristic of the mammalian gene product, and (4) the enzyme synthesised by bacteria containing DHFR cDNA is immunologically reactive with antibody made against mouse DHFR.

Note that the clones which express the highest levels of Tc resistance contain an immunologically reactive peptide having a size characteristic of the mammalian DHFR. A peptide of this size could potentially result from proteolytic cleavage of a fused β -lactamase–DHFR protein that was initiated at the β -lactamase ribosomal binding site. This proposal is consistent with the finding of large-sized protein species containing DHFR antigenic sites in extracts from cells that are either Tp^R or Tp^S (Fig. 6). More intriguing, however, is the possibility noted above that the *Pst*I–poly dG sequence constructed at the vector–cDNA junction can act together with the nearby ATG (AUG) translational start codon to bind mRNA to the bacterial ribosome and initiate DHFR peptide chains within the cDNA insert. If this interpretation is correct, initiation of peptide chains within other eukaryotic cDNA inserts may be obtainable in bacteria by use of the same structural relationships that have resulted in expression of the mouse DHFR coding sequence. Additional DNA

sequence analysis and investigation of the protein products encoded by chimaeric plasmids should provide definitive information on this point and should help elucidate further the structural basis for the different levels of Tp resistance expressed by various clones.

Some of the bacterial clones we have isolated produce proteins that react immunologically with antibody to mouse DHFR but which are not biologically active. Our results suggest that an important obstacle to functional expression of mammalian DNA sequences in bacteria has been the development of an assay capable of detecting those clones that possess both a complete coding sequence and the correct nucleotide relationships to allow such expression. The strong phenotypic selection possible in the present experiments has provided an effective means of identifying and isolating expressing clones.

As the cloned coding sequence for mouse DHFR is selectable in higher organisms as well as in bacteria, it constitutes a powerful tool for the construction of eukaryotic cloning vectors, for the isolation of replication regions of eukaryotic chromosomal and extrachromosomal genomes (compare ref. 30), and for the isolation and characterisation of signals that control genetic transcription and translation in variety of species.

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letters to nature

N Galaxies—a new class of X-ray sources

BURBIDGE¹, classified extragalactic objects with bright nuclei into three classes: in order of increasing optical luminosity these are (1) Seyfert galaxies; (2) N galaxies; (3) quasars. Many Seyferts and several quasars have been shown to be X-ray sources. Here we show that N galaxies are also powerful X-ray sources. In fact, all six N galaxies in the 3C radio catalogue with redshifts less than 0.06 (ref. 2) are detected by the first full-sky survey of the Goddard Space Flight Center detectors (A2) on HEAO 1. X-ray emission has also been discovered from a strong Southern Hemisphere radio source, the N galaxy Pic A. Six of the seven objects are classified as broad-line radio galaxies (BLRGs)^{3–5} and one (3C371) is classified as a BL Lac object⁶. These objects are differentiated from the newly discovered class of 'emission line' galaxies⁷ by their high radio flux and extremely broad lines. Before HEAO 1 there was one confirmed N galaxy, 3C120 (ref. 8), and one suggested, 3C390.3 (refs 9, 10). We detect 3C120, strengthen the identification of 3C390.3, and present evidence for X-ray emission from four new sources, 3C111, 3C382, 3C371, and Pic A. We also suggest 3C445 as the identification of 2A2220-022 (ref. 11).

Figure 1 shows 90% confidence error boxes on the positions of these seven galaxies together with 4U (ref. 10) or 2A (ref. 11) error boxes where applicable. The 2–10 keV luminosity of these objects, as well as other relevant information, is given in Table 1. We have also looked for X-ray flux from the next closest N galaxies, PKS0521–36 and 3C227. No significant flux was seen, but the 90% upper limit on the X-ray luminosities (given in Table 2) are consistent with their luminosities being comparable to the seven detected N galaxies. Except for quasars, these N

galaxies are the most luminous class of compact X-ray sources yet detected.

Grandi and Osterbrock have classified radio galaxies spectroscopically as BLRG or narrow-line radio galaxies (NLRG) and have demonstrated a high degree of association between the classification 'broad-line' and the morphological type 'N'. In marked contrast to the situation for BLRGs, only 3C405 (Cyg A) of the five NLRGs listed by Grandi and Osterbrock, with redshifts less than 0.06, is a detected X-ray source. 3C317 is confused with Ab2052, a distance class 3 cluster, and so its luminosity is poorly determined. Upper limits on the flux from the other three NLRGs are given in Table 2. The deduced upper limits on the luminosities are considerably less than average X-ray luminosities for BLRG of $\sim 3 \times 10^{44}$ erg s⁻¹. Note that of the detected N galaxies, the one with the narrowest emission lines, 3C371, has the smallest X-ray luminosity.

The lack of X-ray emission from NLRGs contrasted to the virtual certainty of X-ray emission from BLRGs is similar to the distinction between type 1 and type 2 Seyferts, that is, none of the ~20 known X-ray emitting Seyfert galaxies are classical type 2 Seyferts. Grandi and Osterbrock have drawn attention to the optical spectral similarities and differences between BLRGs and type 1 Seyferts, and NLRGs and type 2 Seyferts, respectively.

The fact that many of these N galaxies possess compact radio components suggests that the synchrotron self-Compton process (SSC)¹² may be the principle cause of the X-ray emission. For 3C390.3, the best studied of these objects, a SSC model predicts a physical size to the X-ray emitting region of ~0.1 pc and a time scale for X-ray variability of months¹³. The detection of 3C390.3 by Uhuru and Copernicus at considerably higher flux levels and an Ariel 5 upper limit on 3C382 (ref. 14) ~20% lower than the present detection indicate possible variability in

Table 1 Positive detection of N galaxies

Object	X-ray name	Z	L_x , HEAO* (2–10 keV)	Previous L_x	Notes
3C111	H0415+37	0.0485	5.6×10^{44}	—	Near 3U0430+37
3C120	2S0430+05	0.033	1.4×10^{44}	2.3×10^{44} (ref. 7)	SAS-III position
3C371	H1807+69	0.0506	0.5×10^{44}	—	BL Lac object
3C382	H1832+32	0.0586	5.5×10^{44}	$\leq 4.5 \times 10^{44}$ (ref. 14)	—
3C390.3	4U1847+78	0.0569	2.4×10^{44}	4.4×10^{44} (ref. 9) 12×10^{44} (ref. 10)	Variable ⁹
3C445	2A2202-02	0.0568	4.5×10^{44}	7×10^{44} (ref. 11)	Variable?
Pic A	H0519-45	0.0342	0.8×10^{44}	—	—

* Luminosity converted assuming all spectra are like that of 3C390.3 and $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$. Statistical errors are $\sim 20\%$ for weakest source.

this class of object. However, results from the second complete HEAO 1 sky scan will provide a better test of variability in these objects because of the difficulty in comparing the fluxes of these weak sources between different satellite experiments. Preliminary analysis of data from the first HEAO 1 survey indicates no variability ($\pm 50\%$) for these sources on a day-to-day basis.

Alternatively, Fabian¹⁵ has suggested that the X rays are produced by gas clouds colliding with velocities of the order of the Doppler width of the broad lines. This would account for the strong selection seen between BLRGs and NLRGs and the hard spectrum seen in these objects. (Detailed spectral analyses are in progress and will be reported elsewhere.) Only simultaneous radio and X-ray observations similar to that already performed for Cen-A¹⁶ can distinguish between these two mechanisms.

Further HEAO 1 observations will provide the opportunity to determine the detailed spectra of these objects and their variability on a time scale of months. Also, further BLRGs, when they are discovered optically, may be identified with some of the ~ 20 new¹⁷, weak, unidentified sources discovered with the HEAO 1 A2 experiment.

Table 2 90% Confidence upper limits

Name	Z	L_x (90%)	Notes
3C98	0.0306	$< 0.5 \times 10^{44}$	NLRG
3C178	0.0073	$< 0.02 \times 10^{44}$	NLRG
3C192	0.0596	$< 1.0 \times 10^{44}$	NLRG
3C227	0.0861	$< 3.3 \times 10^{44}$	BLRG
3C317	0.0351	$< 1.6 \times 10^{44}$	NLRG confused with distance class 3 Abell cluster 2052. New source H1513+07
PKS0521-36	0.061	$< 1.5 \times 10^{44}$	N galaxy

We thank A. Fabian for suggesting 3C382 as an X-ray source and S. Pravdo and J. Swank for their help. F.E.M. and R.F.M. are NAS/NRC research associates. The A2 experiment on HEAO 1 is a collaborative effort led by E. Boldt of GSFC and G. Garmire of CIT with collaborators at GSFC, CIT, JPL and UCB.

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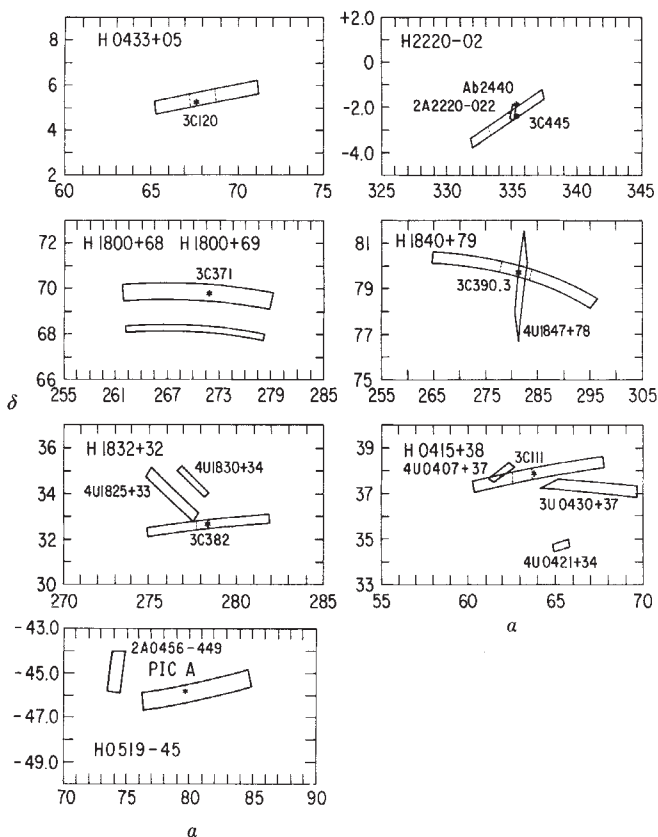


Fig. 1 HEAO 1 A-2 90% confidence error boxes. Other error boxes indicated where applicable. The asterisks indicate the candidate object. The dashed lines indicate 90% confidence error boxes under the assumption that the source intensity did not vary during the observation. In the case of 3C371 there is an additional new source H1800+69 indicated. On H220-02 the smaller box is 2A220-022.

The solar wind at the turn of the century

GEOMAGNETIC ACTIVITY is driven by the solar wind, so information on the past history of the wind can be derived from studies of geomagnetic indices¹⁻³. Comparison of geomagnetic activity and sunspot number over the past hundred years shows that although both vary with the 11-yr solar cycle, they do so with characteristically different patterns. Geomagnetic activity rises and falls with about the same amplitude over each 11-yr solar cycle, but these variations seem to be superposed on a longer-term cycle of similar amplitude. Consequently there is a long-term variation in the 11-yr cycle minima as well as maxima. In contrast, in the sunspot number record the long cycle appears as changing amplitudes of the 11-yr cycles, with sunspot number returning to near zero at each 11-yr minimum. As geomagnetic activity reflects solar wind conditions, it is possible to extrapolate backwards in time the present-day solar wind measurements taken near the long-cycle maximum and we estimate here that around 1900, near the long-cycle minimum, either the average solar wind speed was as much as a factor of two lower, or the average southward component of the solar wind field was as much as a factor of three smaller, or both parameters were significantly less than at present. Thus, in the not too distant past the solar energy output by means of the solar wind was different from what it is now.

That the solar cycle variations of sunspot number and geomagnetic or auroral activity are different has been known for some time. Fritz⁴ first noted that the auroral cycle lags the sunspot cycle. More recently, spectral analyses of the magnetic activity index A_p and sunspot number show that both series have the same strong line at 10.2 yr and that the lag noted by Fritz is of 18 months duration⁵. The shapes of the two solar-cycle variations are also different. Magnetic activity tends to have a secondary, post-sunspot-maximum peak which is associated with recurrent storms⁶.

The difference between geomagnetic activity and sunspot number that we discuss here concerns the pattern of the minima from cycle to cycle. Figure 1 shows the solar-cycle variations of yearly averages of sunspot number and aa index of geomagnetic activity in the top and middle panels, respectively, for those cycles from the 1900s to the 1960s where the $\langle aa \rangle$ minima

Fig. 1 Yearly means of sunspot number, the aa index, and residual aa during the rising years of the long cycle.

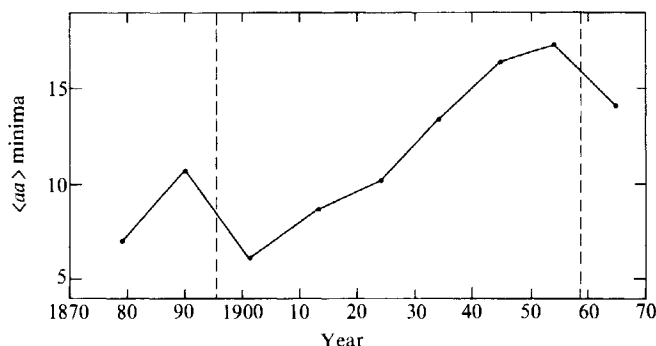
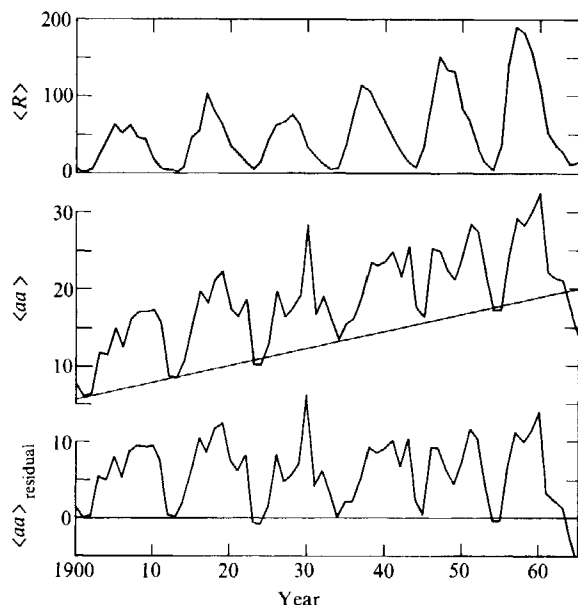


Fig. 2 Solar cycle minima of yearly means of aa . Points between dashed lines increase almost linearly.

increase almost linearly in time. The total increase is about 10 units, an amount typical of the change in $\langle aa \rangle$ over a single solar cycle. The slope of the increase is so steep that $\langle aa \rangle$ at the 1910 maximum is about the same as $\langle aa \rangle$ at the 1954 minimum. In striking contrast is the behaviour of the average sunspot variation. Over the same period the maxima increase by about 120 units; but at each solar minimum the sunspot number returns to near zero.

The increase in sunspot maxima is part of the well-known '80-yr' sunspot cycle, and apparently the rise in the $\langle aa \rangle$ minima is the geomagnetic activity or solar wind manifestation of this same cycle. Brown⁷ has recently shown that although the sunspot number falls to very low values at minima, the 80-yr cycle is also clearly present in the time variation of the minima. In addition, he found that the phase difference between the variations of the minima and the maxima is such that the level of any given minimum is a good predictor of the level of the subsequent maximum.

Figure 2 shows the minima of $\langle aa \rangle$ as a function of time. A root mean least squares fit to the six points between 1900 and 1960 is given by

$$\langle aa \rangle_{\min} = 0.22(T - 1900) + 5.70 \quad (1)$$

with a correlation coefficient of 0.99. The variation of these six points is remarkably linear. The possibility that the linear increase is caused by a systematic error in $\langle aa \rangle$ owing to some change occurring at one of the two stations used to determine the daily aa can quickly be ruled out because almost identical increases occur at the two antipodal stations (Melbourne and Greenwich) used to derive the index. The linear increase is apparently the result of a change in the driver of geomagnetic activity—the solar wind.

Because in the case of geomagnetic activity the 11-yr cycle appears to be superposed on or added to the long-term cycle, the pattern of the 11-yr cycle can be separated from the long-term cycle by defining a quantity $\langle aa \rangle_{\text{residual}}$ given by

$$\langle aa \rangle_{\text{residual}} = \langle aa \rangle_{\text{measured}} - \langle aa \rangle_{\min} \quad (2)$$

where $\langle aa \rangle_{\min}$ is defined by equation (1). The values of $\langle aa \rangle_{\text{residual}}$ are shown in the lower panel of Fig. 1. The important point to note is that the amplitudes of the cyclic changes are all about the same. The residual $\langle aa \rangle$ averaged over each cycle varies only from a high of 7.2 for the 1910s to a low of 5.8 for the cycle beginning in 1924. The shapes of the solar cycle variations of residual $\langle aa \rangle$ are almost square. Post-sunspot-maximum activity peaks are clearly present but less pronounced relative to the first peak in each cycle as compared to the solar cycle variations of $\langle aa \rangle$ in the middle panel. The first peak does not necessarily occur at sunspot maximum, but, on the average, precedes maximum. In the two solar cycles preceding those shown in Fig. 1, the first peaks are well pronounced and also occur before sunspot maximum (see Fig. 4 of Mayaud⁸). Note that these findings are not consistent with the usual association of first peaks with flare-induced geomagnetic storms, which are expected to occur most frequently at sunspot maximum.

Following the suggestion of Hirshberg⁹ that the 11-yr geomagnetic activity cycle, which is so different from the sunspot cycle, may well represent an 11-yr cycle of the solar wind, N.U.C. *et al.*¹⁰ found a remarkable similarity between the variations of geomagnetic activity and solar wind speed over the past solar cycle. To provide good correlations on both short and long time scales, they derive an empirical relationship between geomagnetic activity and the product of the square of the solar wind speed V and the southward component of the interplanetary magnetic field B_s . With a relationship of this kind we can estimate what the solar wind speed and/or magnetic field intensity may have been in 1901.

The regression line of $\langle aa \rangle$ on $\langle B_s \rangle \langle V^2 \rangle$ for data from the past solar cycle is

$$\langle aa \rangle = 1.3 + 4.7 \times 10^{-5} \langle B_s \rangle \langle V^2 \rangle \quad (3)$$

where B_s is measured in units of nT and V in km s^{-1} . The correlation coefficient is 0.9. Equation (3) was used to draw the curves of constant $\langle B_s \rangle \langle V^2 \rangle$ in Fig. 3 for the maximum and

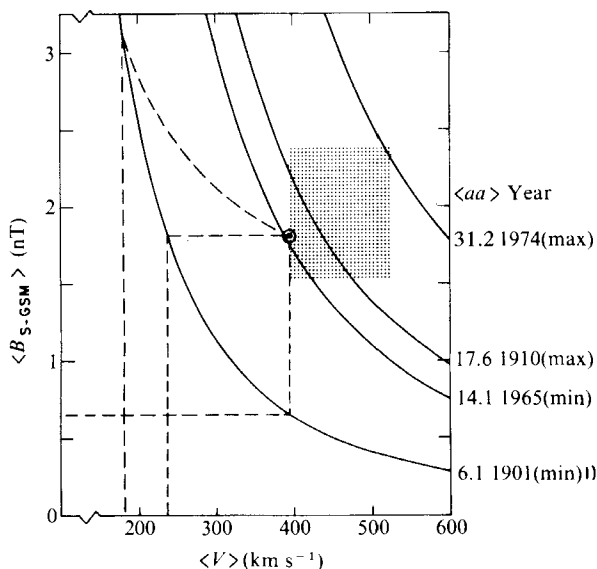


Fig. 3 Curves of constant $\langle B_s \rangle \langle V^2 \rangle$ for the values of $\langle aa \rangle$ listed in the right margin. Shaded area indicates ranges of yearly averages of observed values. Dashed lines extrapolate variables from the 1965 minimum to the 1901 minimum.

minimum $\langle aa \rangle$ for solar cycle 14 beginning in 1901, and for cycle 20 beginning in 1965. Cycle 20 occurs just after the peak in the long cycle, and cycle 14 occurs at the long-cycle minimum. Thus, the overlap in the areas covered by these two cycles in Fig. 3 is small. Cycle 19, with an $\langle aa \rangle$ maximum of 32.9 and minimum of 17.3, would give essentially no overlap; but cycle 20 is plotted in Fig. 3 because it begins the era of spacecraft measurements. The ranges of observed $\langle B_s \rangle$ and $\langle V \rangle$ are indicated by the shaded rectangle, and the encircled datum point marks the averages $\langle B_s \rangle = 1.82$ nT and $\langle V \rangle = 395$ km s^{-1} for the $\langle aa \rangle$ minimum year 1965. Note that the observed $\langle B_s \rangle$ for 1965 is not the lowest observed value, as indicated by the shaded region, which extends down to $\langle B_s \rangle = 1.53$ nT. This lowest value is an average from 1964, the year of sunspot minimum; its uncertainty is rather large, since only about one month of hourly averages are available from 1964¹¹. Interestingly, if curves were drawn for the five $\langle aa \rangle$ minima recorded prior to 1933, none of them would intersect the shaded region of observed solar wind values.

If in one extreme we assume that the solar wind speed in 1901 was the same as during $\langle aa \rangle$ minimum of the recent cycle, it follows that the magnitude of $\langle B_s \rangle$ was about 0.65 nT, as indicated in Fig. 3 by the intersection of the dashed line at $\langle V \rangle = 395$ km s^{-1} and the 1901 curve. This value of $\langle B_s \rangle$ is about a factor of three smaller than $\langle B_s \rangle$ for the 1965 $\langle aa \rangle$ minimum.

As mentioned by Russell², a reduced southward component can result either from a reduction in the intensity of fluctuations relative to the field magnitude, since an undisturbed solar wind has no significant southward component in the solar equatorial plane, or, if that ratio is constant, from a reduction in the field magnitude. Thus if the ratio of the southward component to the field magnitude was the same in 1901 as at present, then the field magnitude may have decreased by as much as a factor of three.

In the other extreme, we can assume that the solar wind field remained constant, and the speed decreased to produce the 1901 $\langle aa \rangle$ minimum. If $\langle B_s \rangle$ remained at the 1965 value of 1.82 nT, then the dashed line at that value in Fig. 3 indicates that the solar wind speed average was 237 km s^{-1} in 1901. A more physically correct treatment of this extreme is to assume that the field magnitude at the Sun and the ratio of $\langle B_s \rangle$ to the field magnitude at 1 AU remained constant. In this case, the increase in field magnitude with decreasing speed at 1 AU must be taken into account. The resulting dashed curve in Fig. 3, which was plotted from Parker's¹² equations, indicates that under these assumptions $\langle V \rangle$ was as low as 180 km s^{-1} . This is indeed a low value for a yearly average in comparison to solar wind measurements of recent times, where hourly averages of ~ 200 km s^{-1} are rare. These values for speeds and field intensities are extremes, and it is more likely that both quantities changed over the 64-yr period.

Although photographs of the corona during eclipses at the turn of the century are available, the plates are not calibrated, so comparisons to the present day corona cannot be made. However, in this study we have shown that there were considerable changes in the solar wind over the past century. This implies some change in the state of the solar corona itself.

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Power harmonic radiation enhancement during the sudden commencement of a magnetic storm

THE enhancements of power harmonic radiation that occurred at the same time as a sudden commencement of a geomagnetic storm (ssc) and with geomagnetic pulsations that followed the ssc are described and discussed here. The power harmonic radiation was detected by an ELF-VLF wave receiver with two loop antennas and a vertical whip at Riverton, while geomagnetic variations were observed by three induction

magnetometers distributed at Thompson (55.9° N, 97.4° W), Riverton (51.0° N, 97.0° W) and Star Lake (50.1° N, 95.2° W), Manitoba, Canada. The power harmonic enhancements are accounted for in terms of the geomagnetically induced telluric current that was possibly shunted through transformer neutrals and a transmission line of a power system, giving rise to a near saturation of the transformer core material. The important role which the enhanced power harmonics may have in stimulation of emissions in the magnetosphere is considered.

During a joint IMS campaign for aurora/ULF-VLF waves in Manitoba, Canada, from 10 to 24 September 1977, a magnetic storm broke out on 21 September with an ssc. A generally accepted concept of an ssc was first presented in 1933¹ which in terms of hydromagnetics is described as a rapid compression of the Earth's magnetosphere due to an abrupt increase in the solar wind kinetic pressure². This specific event is ssc* in type which is characterised by a prominent negative kick on the magnetic horizontal (N-S) component before the compressional (positive) increase. Induction magnetograms at three stations, Thompson, Riverton and Star Lake indicate that the negative kick (southwards deflection) started 20.45.00 UT simultaneously at all three stations. The negative phase lasted until 20.45.47 at Thompson, 20.45.33 at Riverton and 20.45.30 at Star Lake. Following the negative phase, the compressional increase (northwards deflection) of the horizontal component took place during the intervals 20.45.47–20.47.30 at Thompson, 20.45.33–20.46.25 at Riverton, and 20.45.30–20.46.17 at Star Lake as can be seen in the lower panel of Fig. 1. This positive phase was followed by damped oscillations. At Riverton, the damped oscillation took a period of approximately 2 min and continued until 20.53.00. The ssc* and the subsequent damped oscillations in the Earth's magnetic field gave rise to two effects on VLF emissions which were recorded at Riverton concurrently with the ULF magnetic observation using a receiver system for finding the arrival direction³.

One of the two effects concerns natural VLF chorus emissions. This effect is already known and has been explained in terms of increases in gyrofrequency of suprathermal electrons and in the anisotropy factor of their distribution in velocity space during the course of a magnetospheric compression⁴. Bandwidth broadening, increase in mean frequency and enhancement of band-integrated intensity—the changes characterising the ssc effect in chorus as seen in the spectrogram in the upper panel of Fig. 1—are reasonably well understood in terms of the changes in the two parameters mentioned above.

The second effect, which is the main one considered here, is

enhancement of harmonics in commercial electric power. As the spectrogram shows, the intensities of the power harmonics increased almost concurrently with the distinctive deviations on the induction magnetograms. The main harmonic components before the ssc were the 3rd (180 Hz) and the 9th (540 Hz), both being relatively weak. At the onset of the negative kick, even harmonics came out: the bands of the 6th (360 Hz) and 12th (720 Hz) harmonics were considerably intensified. The pre-existing bands, the 3rd and 9th harmonics, seem to have been enhanced also. It is interesting that the harmonics appeared at multiples of 180 Hz as if it were the fundamental frequency. This suggests that they were generated in a three-phase a.c. transmission system. These bands then gradually decreased in intensity towards 20.45.45. They were enhanced again in a time interval 20.45.50–20.46.45 during which the harmonics up to 18th (1,080 Hz) were excited. After this, their intensity was increased again and again, for example, from 20.47.00 to 20.47.12, from 20.47.25 to 20.48.33 and so forth as shown in Fig. 1.

The initiation of the first enhancement of the harmonics (especially 6th) coincided with the initiation of the negative kick at Riverton where the VLF recording was carried out, but their decay came out about 10 s behind the positive turn of the magnetic deflection. The second enhancement then began about 15 s behind the positive turn and continued until about 20 s behind its negative turn at Riverton. The third and the fourth enhancements also occurred 15–20 s behind the corresponding deflections of the horizontal component at Riverton.

The above facts strongly suggest that the harmonics were generated in a power system near the VLF observation site at Riverton due to the effects of the ssc. One possibility is that the ssc-induced electric field gave rise to an electric current in a circuit consisting of ground–transformer A–transmission line–transformer B–ground. The current intensity must have had a slow temporal variation with a time scale comparable to that of the magnetic field variations detected by the induction magnetometers. The current probably gave rise to a quasi-d.c. bias to the hysteresis loop of the core material of the transformers, with probable magnetisation to near saturation with resultant non-linear distortion of the current waveforms⁵. Quasi-d.c. electric currents between a transformer neutral and ground have been recorded. Enhanced currents are referred to as solar-induced currents (SICs) in electrical engineering⁶ and were found to be as large as 100 A, during severe magnetic storms.

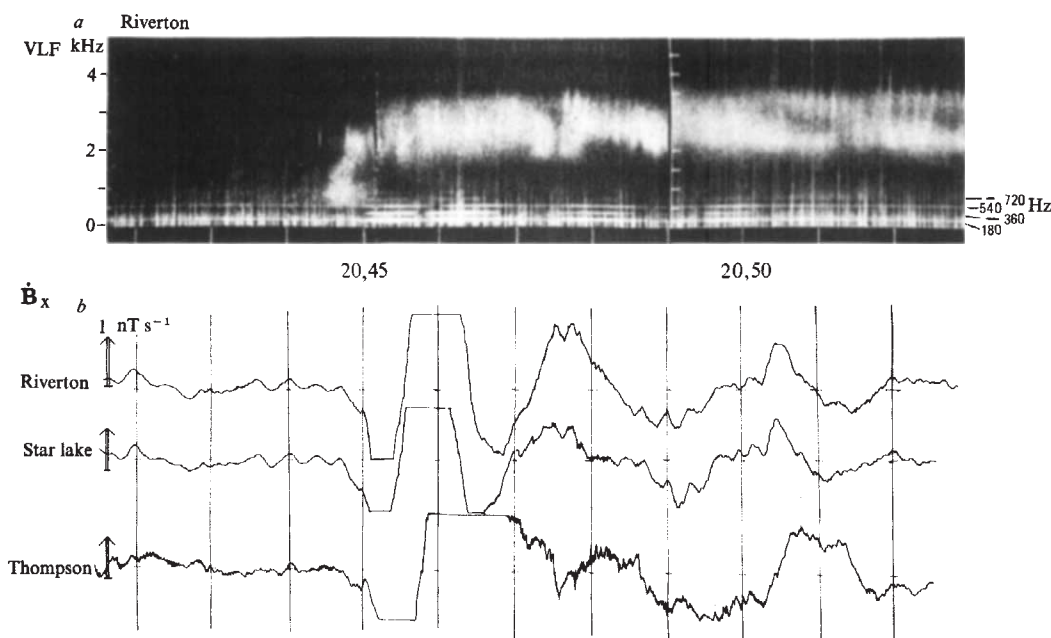


Fig. 1 A frequency-time spectrogram of chorus emission and power line harmonics (a), along with induction magnetograms of the magnetic horizontal component recorded at Riverton, Star Lake and Thompson (b). The ELF-VLF signals (magnetic component) were received on a loop antenna whose plane lay in that of the magnetic meridian. The power harmonics are seen as horizontal lines in the low frequency band below 1 kHz. Their enhancements start coincident with the initiation of the rapid negative deflection of the magnetic horizontal component.

The non-linearity in magnetisation of the transformer core materials is bound to give rise to higher harmonics in power line currents. It has been shown that the third harmonic is dominant among the higher harmonics⁷. According to our analysis, only odd harmonics are excited if the hysteresis loop of the transformer core materials has a point of symmetry, whereas both odd and even harmonics are excited in the absence of point-symmetry. In the absence of d.c. bias, the hysteresis loop of a ferromagnetic material has a point of symmetry; however, the symmetry is generally lost if a d.c. bias is applied. This readily explains why odd harmonics only (3rd and 9th) existed before the ssc whereas both odd and even harmonics (6th, 12th and 18th) were generated corresponding to the rapid magnetic fluctuations of the ssc.

The foregoing cause of SIC-induced harmonics suggests that the 10–20-s delay of the enhancements of the harmonics after the beginnings of the magnetic fluctuations on the horizontal component at Riverton may be attributed to the hysteresis of the transformer core, as the induced current must be reversed between one harmonics enhancement and the next. Possible phase differences between magnetic fluctuations and telluric currents may also be responsible for the delay.

It is appropriate to check whether the ssc-induced telluric current can be strong enough to bring the transformer cores to near saturation. If we assume that the power line is straight and l km in length, flow-pattern of the telluric currents is likely to be affected in an area of order of l^2 km². Considering a square whose side length is l km, the total current flowing across the square's side perpendicular to the power line can be estimated as follows. An abrupt magnetic field change of 1 nT is estimated to give rise to telluric currents with depth-integrated strength of ~ 1 A km⁻¹. The size of the ssc under consideration is estimated to be ~ 50 nT. Consequently, the strength of the telluric current is ~ 50 A km⁻¹. Assuming that l is 300 km, the total current flowing across the perpendicular side is $\sim 15,000$ A. A very small portion of this current, if shunted by the power line, can affect transformer cores considerably.

The observation-interpretation of the ssc-enhanced power harmonics leads to two points of interest: one scientific and one industrial. The scientific point concerns VLF chorus emissions. Chorus emissions are known to increase during magnetic substorms. This is generally understood in terms of the increased population of suprathermal electrons in the magnetosphere⁸. However, our investigation indicates that the enhancement of power harmonics during a magnetic storm may, itself, have an important role in giving rise to enhanced stimulation of emissions in the magnetosphere. The seeding of the harmonic waves is known to increase the emission activity^{8–12}, and our observation shows that the seeding process itself is enhanced during magnetic disturbances. Note that enhanced seeding of power harmonic waves means not only the increase in wave amplitude but also enrichment in spectral content, extension to higher frequencies and occurrence of even harmonics. The industrial point is that suitably distributed VLF receivers and induction magnetometers are expected to be useful for monitoring SICs in power systems. Observation of power harmonic waves by VLF receiver is expected to yield quantitative information on strength of SICs. SICs are likely to be caused by various types of magnetic variations: ssc, magnetic pulsations, polar substorm and magnetic storms. Identification of the particular type can be made through observation by induction magnetometers. Understanding the nature of SICs is expected to help find ways of minimising their undesirable effects on power generation, transmission and distribution.

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Thermoluminescence and terrestrial age of the Estacado meteorite

THE terrestrial age of a meteorite is the number of years which has elapsed since the meteorite fell to Earth. The terrestrial ages of most meteorites are not known directly, as most are 'finds' rather than observed 'falls'. Here we discuss the various methods which have been suggested to determine the terrestrial age of the 'finds', and these may be conveniently divided into two categories: (1) those which use the decay of spallation-produced radioactive isotopes^{1–4}; (2) those which use the thermal drainage of thermoluminescence (TL) from the material^{5,6}.

Boeckl¹ has calculated the terrestrial ages of 19 stony meteorites from a derivation of their radiocarbon content. Among the meteorites studied was Estacado (H-group chondrite) for which Boeckl calculated a terrestrial age of $6,800 \pm 3,000$ yr. Recently, McKeever and Durrani⁷ have carried out some TL studies of Estacado, the results of which are not consistent with such a large terrestrial age. After a meteorite falls, it is shielded from cosmic rays by the Earth's atmosphere. The TL induced in the meteorite by cosmic-ray bombardment in space will thus slowly drain from the meteorite as it rests on the Earth's surface, the high-temperature ($\approx 400^\circ\text{C}$) region of the TL glow decaying much more slowly than the low-temperature ($\approx 200^\circ\text{C}$) region. The high-temperature region of Estacado's TL glow seems to be far too high to be consistent with a terrestrial age of 6,800 yr.

Figure 1 shows the high-temperature (HT) TL of 14 meteorites of known terrestrial age (that is, 'falls') and the high-temperature TL of five 'finds'. The TL is plotted either against known terrestrial age, or against deduced age from ¹⁴C

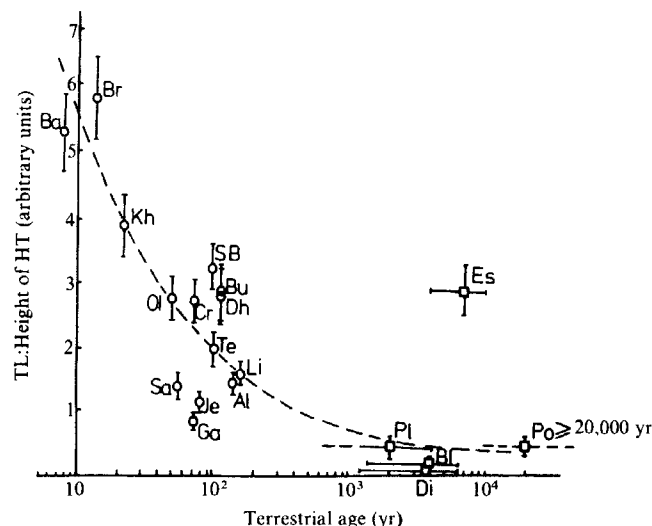


Fig. 1 The decay of the intensity of the high-temperature TL region from 14 'falls' (○) and five 'finds' (□). The ^{14}C age for Potter is from Suess and Wänke⁴. The uncertainty in the TL is estimated from the standard deviation of the high-temperature TL within a sample, based on 75 measurements of the natural TL in Estacado. The dotted curve is an estimation of the decay curve. Ba, Barwell; Br, Bruderheim; Kh, Khanpur; SB, Soko Banja; Bu, Butsura; Ol, Olivenza; Cr, Crumlin; Dh, Dhurmsala; Es, Estacado; Te, Tennasilm; Li, Limerick; Al, Aldsworth; Sa, Saratov; Je, Jelica; Ga, Gambat; Pl, Plainview; Bl, Bluff; Di, Dimmit; Po, Potter.

measurements^{1,4}. Clearly, Estacado is 'anomalous' in that the intensity of the high temperature TL is not consistent with a ^{14}C age of 6,800 yr. Indeed, the TL data suggest a true terrestrial age of 100 yr.

Further evidence is available to support a terrestrial age ≈ 100 yr for Estacado. First, its appearance is one of a reasonably 'fresh' fall. Besides rusting on the surface, the interior of the meteorite was very fresh looking (a pale grey) which, when the meteorite is compared with meteorites such as Dimmit or Potter, implies that it has a much lower terrestrial age than 6,800 yr.

Second, there is some strong circumstantial evidence to indicate that Estacado fell in 1882. Howard and Davison⁸ have given a detailed account of the find of Estacado. In 1882, a bright fireball was seen by the residents of the colony of Estacado, Hale County, Texas. The meteor was seen to fall north-west of them. In 1883 several cowboys came across the meteorite in the vicinity of the conjectured fall of the meteor. As this is a stoneless region, the inhabitants considered the meteorite to have been the cause of the fireball seen in 1882.

Viewed in the light of the account reproduced by Howard and Davison, the TL data clearly support the view that Estacado's terrestrial age is only ≈ 100 yr and not several thousands of years as deduced by Boeckl.

This discussion of Estacado's terrestrial age suggests that a useful check of a deduced ^{14}C age would be to carry out some TL measurements in order to check the consistency of the ^{14}C age with the TL properties. Similar inconsistencies have been highlighted in earlier work⁵.

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An explosive volcanic eruption in the Southern Hemisphere in 1928

SEVERAL climatologists^{1–3} have compiled lists of volcanic eruptions which are thought to be of meteorological and climatological importance. Although these lists have been criticised for being incomplete⁴, subsequent authors^{5,6} have used them in studies designed to reproduce and explain the climatic history of the Northern Hemisphere. I report here additional information on two volcanic eruptions which may be climatologically significant but have generally not been considered in climate studies. By clarifying the volcanic chronology, a better test of the hypothesis that volcanoes cause part of the observed climate change can be made.

From measurements of solar radiation by the Smithsonian Astrophysical Observatory made at Mt Montezuma, Chile, Mt Brukkaros, South-west Africa, and Table Mountain, California, there is evidence for an increase in atmospheric turbidity in 1928 which can be best attributed to a volcanic eruption which ejected aerosols into the stratosphere. Paluweh (8° 19'S, 121° 42'E) in the East Indies, which caused tidal waves with its eruption in 1928, is the major cause of the decrease in atmospheric transmission at this time, although Reventador (0° 05'S, 77° 40' W) in Ecuador which also erupted in 1929 may have contributed to this decrease. Radiation records at Washington, D.C., Madison, Wisconsin, Lincoln, Nebraska, and Blue Hill, Massachusetts also show decreases in atmospheric transmission⁷. Modern climatologists have generally overlooked the volcanic eruption of Paluweh in 1928, although this was probably one of the largest eruptions between that of Katmai in 1912 and Agung in 1963.

The Astrophysical Observatory of the Smithsonian Institution made pyrheliometric observations of the normal incidence irradiance of the Sun at Mt Montezuma, Chile (22° 40' S, 68° 56' W) from 1920 to 1955. These measurements were made with silver disk pyrheliometers and Angstrom pyrheliometers at selected air mass values. Many measurements were made at sea level air mass values of 2.0 and 2.5 and are tabulated by Abbot and Aldrich⁸ and Aldrich and Hoover⁹. The true air mass values are 1.44 and 1.80 for the mean station surface pressure.

The ratio of the pyrheliometric measurements at air mass 2.5 to that at 2.0 provides a relative measure of atmospheric transmission¹⁰ which is independent of the pyrheliometer calibration. Ratios are calculated on each day for which the pair of observations are available. Monthly means are calculated from the daily values. For those months with no data, the average value of all the months with the same name is used. The resulting time series is then smoothed by using a 13-month running mean average to remove seasonal variations. The resulting transmission curve is shown in Fig. 1.

Most of the features of this curve can be explained by known causes. The low transmission values before 1926 are caused by the volcanic eruption of Puyehue, Chile (40° 35' S, 72° 08' W) in December 1921 (refs 11, 12). The decrease in atmospheric transmission in 1932 was caused by the eruption of the Quizapu crater of Cerro Azul (35° 39' S, 70° 46' W) in Argentina in April 1932. A volcanic cloud 15 km in height was observed¹². This stratospheric event was observed throughout the Southern Hemisphere¹³ and ash fell over much of South America¹⁴. From Fig. 1 it can be seen that it took 2–4 yr for the atmosphere to return to normal transmission. This behaviour is characteristic of a violent volcanic eruption in which aerosols are injected into the stratosphere. After 1950 the atmospheric transmission decreases because of local contamination by a copper smelter at the Chuquicamata mine located north of the station. This local

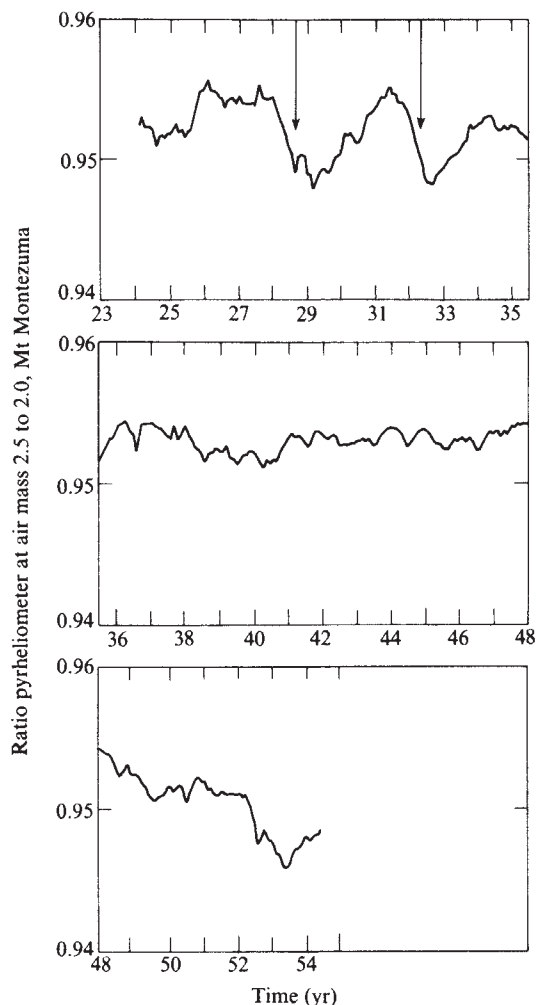


Fig. 1 The 13-month running mean values of the ratios of the pyrrehliometer measurements at air mass 2.5 to that at air mass 2.0 at Mt Montezuma, Chile. It is a measure of the relative atmospheric transmission. The increase in optical depth for air mass 1 after the eruption of Paluweh in 1928 averaged about 0.02 for 6 months after the eruption at Mt Montezuma. The times of the eruptions of Paluweh (1928) and Quizapu (1932) are indicated by arrows. Because running means are used, the apparent atmospheric transmissions seem to start decreasing about 7 months before the date of the eruption.

contamination becomes particularly evident in late 1952 with the opening of a major smelter.

Another decrease in atmospheric transmission is observed starting in 1928. The decrease and subsequent slow rise in atmospheric transmission is very similar to the event of 1932. It seems to be a stratospheric volcanic event in July or August 1928, but it is not mentioned in the usual lists of explosive volcanoes¹⁻³. The Smithsonian observations of the aureole showed an increase in brightness, and the pyrrehliometric measurements a decrease in intensity at this time in a manner similar to that in the period after the Quizapu eruption. There was no appreciable change in total precipitable water. The measured solar constants of the Smithsonian Astrophysical Observatory at Mt Montezuma, Table Mountain and Mt Brukaros also decreased at this time which is an indication of increased atmospheric turbidity because of errors in their reduction scheme¹⁵. Clearly, the decrease in atmospheric transmission in 1928 was caused by an increase in the stratospheric dust loading both because of the long period of decreased atmospheric transmission and because it was observed worldwide. Indeed, Stormer¹⁶ photographed stratospheric dust clouds in Europe in January 1929 which may have originated from the 1928 volcanic eruption.

Although several volcanoes erupted in the period 1927-29, the eruption of Paluweh in the Isle Flores or the East Indies was the primary cause of the increase in turbidity observed at Mt Montezuma. Paluweh was active from 4 August to 25 September 1928 (ref. 17) and was the only violent volcanic eruption at this time. The eruption was accompanied by a tidal wave and the creation of three craters with diameters 80-600 m wide. The decrease in atmospheric transmission at Mt Montezuma was observed immediately after this eruption and continued for about 2 yr.

Reventador (0°05' S, 77°40' W) in Ecuador erupted in 1929 with ash rains evident in the Andes¹⁸. This eruption may have prolonged the decreased atmospheric transmission and may account for the double-bottomed depression in Fig. 1. Other minor eruptions in 1928 were Slamet (7°14' S, 109°12' E) in Java, Bromo (7°56' S, 112°57' E) in Java, Raung (8°07' S, 114°02' E) in Java, Ili Werung (8°32' S, 123°35' E) in the Lesser Sunda Islands, and Batuk Petarangan (7°10' S, 109°49' E) in Java. Active volcanoes in 1929 include Quizapu, Mt Pelee on Martinique, and Asama in Japan.

Hand⁷ independently confirms that Paluweh was the major contributor to increased turbidity in 1928 and 1929 using the Weather Bureau pyrrehliometer data. His brief mention of an eruption in the Isle Flores, Dutch East Indies, seems to be the only previous comment on a volcanic eruption at this time in the meteorological literature. At Washington, D.C., Madison, Wisconsin, Lincoln, Nebraska, and Blue Hill, Massachusetts, the atmospheric transmission decreased beginning in April of 1929. The Southern Hemisphere autumn is the most favourable time for the transport of stratospheric aerosols across the Equator^{2,19}, hence the delay of 6-8 months of the effect of the eruption of Paluweh on the radiation records in the US. Meinel and Meinel²⁰ found a comparable delay before volcanic dust from the Agung eruption became evident over the US; Paluweh and Agung are located close to each other. From the Smithsonian records, the Lamb dust veil index for this volcano seems to be about 100, thus making it the most important eruption between Katmai in 1912 and Agung in 1963. The decrease in atmospheric transmission in the US after the Paluweh eruption was about 30-40% of that observed after the Agung eruption.

The eruptions of Paluweh in 1928 and Quizapu in 1932 may have had a worldwide effect on the mean surface temperature, as indicated by Fig. 2 taken from Oliver⁵. One year after both eruptions there was a decrease in the mean surface temperature of the Northern Hemisphere of the order of 0.3 °C. Miles and Gildersleeves²¹, have shown that 1 yr after a major volcanic eruption there is on the average a decrease in the mean surface temperature of 0.29 °C and that there is only 'a small probability of the result being due to chance.' Mass and Schneider⁶ find that the depression in surface temperatures 1 yr after a volcanic eruption is significant at better than the 99% confidence level. Humphreys²², Mitchell²³, Yamamoto *et al.*²⁴, and others reach similar conclusions based on experimental data, and Hansen *et al.*²⁵, Baldwin *et al.*²⁶, and others support these observations by theory. Thus, there is reason to believe that the depressed surface temperatures in 1929 and 1933 may have a cause and effect relationship with the volcanic eruptions occurring 1 yr earlier. These drops in temperature may occur by chance but the probability of this happening is low, probably less than 1%. From Budyko²⁷, the year-to-year variation in the Northern Hemisphere annual mean surface temperature is 0.12 ± 0.097 °C for the period 1923-54. Therefore, the probability of a 0.3 °C temperature change being caused by chance alone is about 1% or less using three standard deviations as a guide. Furthermore, the probability of successfully identifying the years when the temperature decreases will occur only from a knowledge of the dates of the major volcanic eruptions is even less. In short, there is a strong suggestion that the volcanic eruptions affect climate 1 yr afterwards but not longer. Further studies of these eruptions and their effect on atmospheric transmission and climate will be needed to confirm these conclusions.

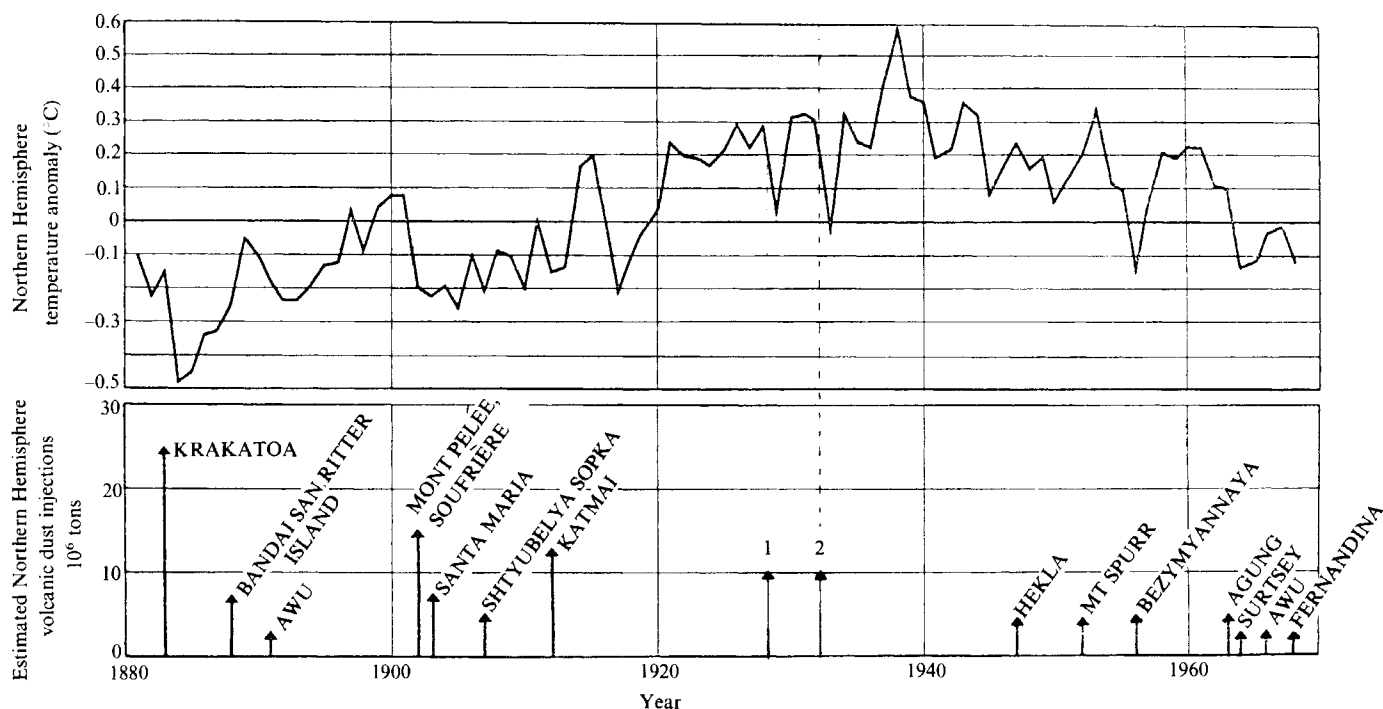


Fig. 2 The Northern Hemisphere temperature anomaly ($^{\circ}\text{C}$) as given by Oliver⁵. Major volcanic eruptions are indicated below. Events 1 and 2 correspond to the volcanic eruption of Paluweh in 1928 and to the Quizapu eruption in 1932. These Southern Hemisphere volcanic events may have affected the Northern Hemisphere temperatures, as can be seen by the decrease in temperatures about 1 yr after the two events. (The arrows for Paluweh and Quizapu are primarily used to indicate the times of their eruptions, not their strength.)

Most climatologists¹⁻³ have failed to include these eruptions in their lists and have included other volcanic eruptions which do not show up in the radiation records. For example, Oliver⁵ omits these eruptions in his analysis of the Northern Hemisphere surface temperatures, but it seems that volcanic dust originating in the Southern Hemisphere can cross the Equator and significantly affect the climate in the Northern Hemisphere. However, we emphasise that not all features of the climatic record can be explained by volcanic eruptions, although certain fluctuations in climate do seem to be caused by this mechanism. The climatology of the Southern Hemisphere is not yet well enough established in the 1920s to determine the effect of the eruption of Paluweh on the climate of the Southern Hemisphere.

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Stimulated resonance between electrical and shear fields by a colloid system

CERTAIN polymer solutions have been shown using a Couette shear dielectric cell¹ to exhibit changes in dielectric properties when subjected to a shear field^{2,3}. For polar polymers which lack flexibility, the action of a rotational shear field alters the time-dependent distribution of dipole orientation which results in changes of permittivity, loss magnitude and relaxation frequency². In quantitative terms these changes seem to correspond well with the theoretical treatment due to Barisas⁴. A distinct difference in the situation occurs when flexible polymers such as poly(styrenes) or poly(*p*-ethoxystyrenes) are subjected to such shear fields. The changes are inconsistent with the magnitude of the polymer dipole and solvent relative permittivity takes on a rather dominant role³. To test the importance of variation of the internal field term on shape changes due to polymer coil distortions, we have studied an organic colloid system: cross-linked poly(ethylacrylate) dispersed in heptane and stabilised with a 'comb' graft copolymer [poly-(12-hydroxy stearic acid) terminated with glycidyl methacrylate and then copolymerised (1:1) with methyl methacrylate]⁵.

This latex system consists of rubbery particles of 0.4 μm average diameter which can presumably shear distort. We observed a resonance (Fig. 1) between the shear field (shear rate G (s^{-1})) and electric field f (Hz). We know of no previous observation of such a phenomenon.

Certain preliminary observations suggest a mechanism for the effect. A study of the frequency dependence of relative permittivity (ϵ') and loss (ϵ'') in the absence of a shear field indicates, as shown in Fig. 2, that the heptane dispersed colloid has a high relative permittivity at low frequency and that at least two loss

processes are present. One occurs with a maximum at such a low frequency ($<10^2$ Hz) that its resolution is not possible with our audio frequency bridge equipment; the other apparently occurs at a frequency $\sim 10^9$ Hz as indicated by spot coaxial line and wave guide measurements. Even attempts to shift the low frequency process completely into the range of measurements ($>10^2$ Hz) by increasing the temperature to 60°C failed, though a partial shift did take place. A further temperature increase was not practicable due to solvent volatility. The large value of the relative permittivity at 10^2 Hz of a 40% dispersion above that of pure heptane (Fig. 2) together with the presence of two loss

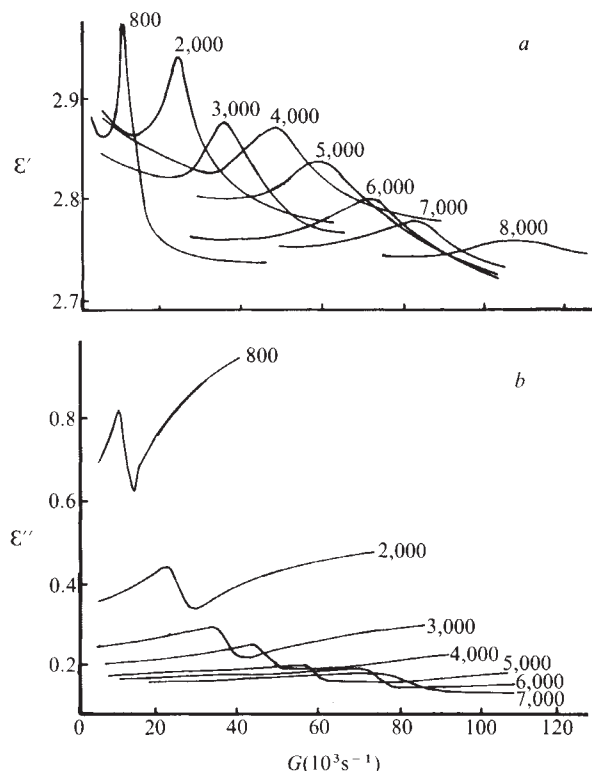


Fig. 1 Resonances in relative permittivity (ϵ') (a) and dielectric loss (ϵ'') (b) for the organic colloid in shear (rate G (s^{-1})). Numbers give polarisation frequency f (Hz).

processes at vastly different frequencies suggest that dissimilar mechanisms are responsible. The high frequency process is probably dipolar whereas that at low frequency is in our view interfacial (Maxwell-Wagner), although a dipolar origin in the poly(ethylacrylate) cross-linked phase cannot be ruled out. We favour an ionic-conductance process due to trace impurity ions, through or over the surface of the particles, promoting interfacial polarisation rather than orientation polarisation because of its low frequency, large magnitude and its agreement with a mechanism described below. Also, at the frequency of this process a bulk conductivity term is present shown by values of ϵ'' exceeding the incremental relative permittivity ($\Delta\epsilon'$).

On the basis of an interfacial polarisation the resonances shown in Fig. 1 originate in a coupling between electric field-induced charge displacement and forced rotation of the particles by the shear field. Indeed, the condition for the position on the shear-rate axis for such maxima (G_{max}) can be predicted to occur when the angular frequencies of polarisation and rotation match. The condition that $2\pi f = G_{\text{max}}/2$ follows if the particles are spherical and rotational diffusion has no influence⁶. Angular fluctuations due to brownian motion for such large particles will be small and even if significant, are not likely to alter the position of the maximum. The neglect of diffusion is therefore justified. It follows that f should be proportional to G_{max} and this is shown in

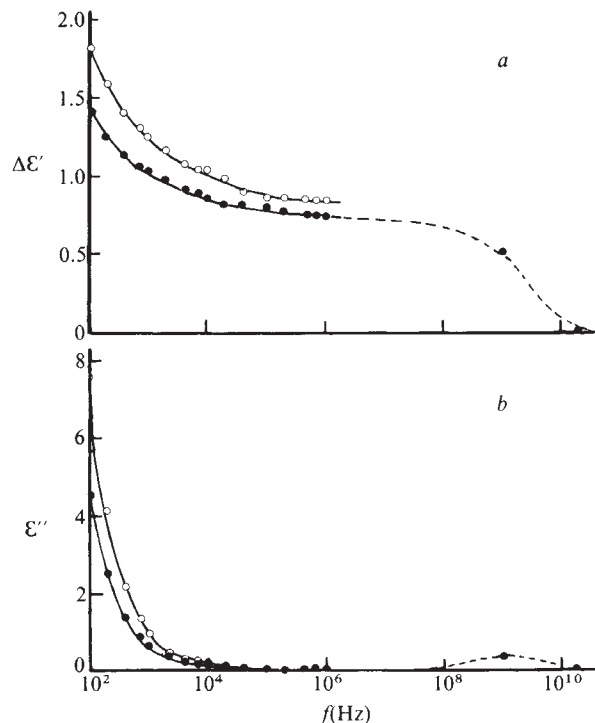


Fig. 2 The increment in relative permittivity ($\Delta\epsilon' = \epsilon'_{\text{solution}} - \epsilon'_{\text{solvent}}$) (a) and dielectric loss (ϵ'') (b) of the organic colloid in heptane. $\bullet$, 30° ; $\circ$, 60° . Dashed line is interpolation based on limited data.

Fig. 3. Also shown in Fig. 3 is the theoretical line of slope $(4\pi)^{-1}$. The slight discrepancies we attribute to inaccuracies in gap measurement. An error of $1.3\ \mu\text{m}$ in the gap measurement of $23\ \mu\text{m}$ would account for the divergent slopes: this is within the limits of the capacitance based measurement¹.

Work is proceeding on this resonance phenomenon and its application to aqueous media. This important class of polymer systems, which includes biopolymers in their natural environment, presents considerable problems in dielectric studies in the kHz-MHz frequency range because of the screening effect of bulk conductance. We have experimentally established with dioxan-water mixtures that bulk conductance in isolation does not give rise to a shear-induced resonance in permittivity. Thus, this resonance phenomenon may possibly be used to decouple

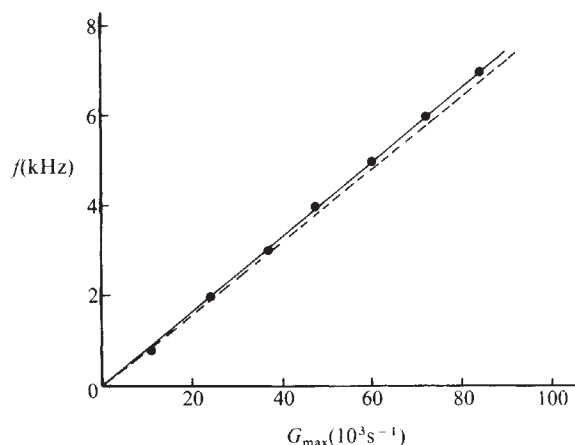


Fig. 3 The dependence of shear rate at maximum resonance (G_{max}) on f . The theoretical dependence of slope $(4\pi)^{-1}$ is shown as a dashed line.

dielectric relaxations, particularly interfacial ones, from the screening effect of bulk conductivity.

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Epitaxial synthesis of diamond by carbon-ion deposition at low energy

THIN epitaxial layers have been grown in vacuum by the controlled deposition of low-energy carbon ions on to heated single crystal diamond substrates by a process described here. Reproducible crystalline growth, up to 10 μm in thickness and up to several mm^2 in area, has been observed on both natural stones and polished diamond plaques¹. Three competing forms of ion-deposited carbon have also been characterised.

Various methods of diamond synthesis have been reported; the most successful involve growth at elevated temperature under very high pressure. Limited success has also been achieved in the metastable region by vapour-phase epitaxy at low pressure², but such procedures are constrained by the spontaneous graphitisation of diamond at high temperatures. At the lower temperatures which have been used, the scale and the quality of crystalline growth are severely limited by the competing formation of other modifications of carbon.

In 1971, Aisenberg and Chabot³ described the production of 'diamond-like' films by the deposition of low-energy carbon ions on a variety of surfaces. The depositions were carried out at room temperature and the substrates did not include diamond. The further characterisation of such films by ellipsometry and X-ray and electron diffraction has been reported by Spencer *et al.*⁴. Although the properties of the deposits were variable, there was evidence of polycrystallinity with definite, but usually very weak, X-ray lines assignable to diamond.

In 1967 J.H.F. described the adaptation of a heavy-ion accelerator for a variety of ion-bombardment studies on semiconductor substrates⁵. These included the study of epitaxial growth through the deposition of low-energy silicon ions on heated wafers of single crystal silicon. Such epitaxy is to be expected and it simply reflects the thermodynamic stability and the established vapour-phase growth behaviour of crystalline silicon. It has been subsequently confirmed in more detailed studies⁶ using low-energy silicon and germanium ions. The situation with respect to carbon is quite different; not only is diamond metastable in such deposition conditions, but it has also been observed that the radiation damage due to ion bombardment can result in irreversible graphitisation⁷.

Nevertheless, some encouraging indications of diamond synthesis were obtained in an initial feasibility study carried out in 1972. Carbon ions with an energy < 100 eV were deposited on to a small area of a heated diamond plaque. The resulting brown layer, ~3,000 Å in thickness, resisted simple abrasion

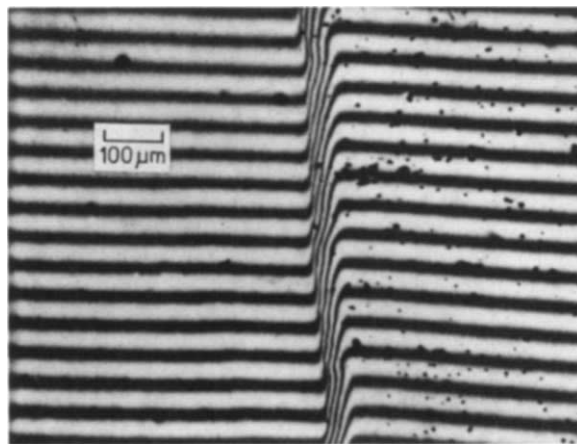


Fig. 1 Interferometric micrograph of an epitaxial deposit on a polished diamond plaque. Note the carbonaceous inclusions in the deposited layer.

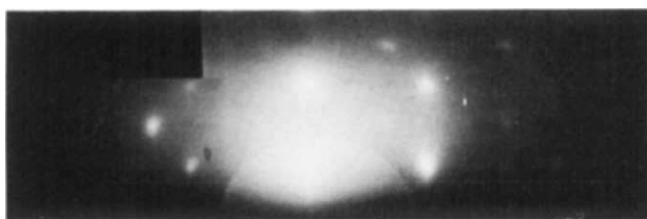
tests and showed evidence of crystallinity when studied by proton channelling. It thus seemed that the very localised dissipation of energy as the ions were stopped on, or very close to, the surface may have assisted epitaxial growth by inducing mobility at an acceptable substrate temperature.

A wide range of experiments was carried out subsequently on a much improved ion-deceleration apparatus⁸ installed on a Harwell/Lintott type of heavy-ion accelerator. These demonstrated that the deposition process is complex and that at least three other forms of carbon can compete with the epitaxial synthesis. It was also found that, at low ion energies, even slight surface contamination affects the reproducibility of deposition. The most consistent results and the best quality of growth were obtained using carbon ions at an energy of around 900 eV on carefully cleaned diamond plaques, heated to about 700 °C. The layers, which were deposited to a thickness of several micrometres at a rate of a few thousand Ångströms per hour, regularly contained a low density of carbonaceous inclusions but otherwise apparently represented continuous epitaxial growth on the crystalline substrate.

An interferometric micrograph of an epitaxial deposit containing inclusions on a polished diamond plaque is shown in Fig. 1. Evidence of crystallinity in such a layer can be seen in the reflection high-energy electron diffraction pattern (RHEED) in Fig. 2. Various other techniques were also used to characterise the ion deposited layers. Finally, on the basis of the accumulated experience with a large number of samples, it was concluded that non-epitaxial carbon could be confidently distinguished and removed by extended polishing with alumina powder followed by controlled air oxidation for periods up to two weeks. The epitaxial growth was totally resistant to such treatment.

It was found that the density of the carbonaceous inclusions could be substantially reduced by rigorous precleaning of the substrate but they were never completely eliminated. They seem to be a lower density form of carbon than diamond and they consequently grow steadily in size as the deposition proceeds. It was thus found that they provide an ultimate limit to the

Fig. 2 Reflection high-energy electron diffraction (RHEED) pattern of an ion-deposited epitaxial layer.



thickness of crystalline growth which can be achieved in a single stage.

In some experiments a continuous smooth deposit with a lower reflectivity and density than diamond was observed in place of the epitaxial growth. The nature of these carbonaceous layers which may have been associated with the use of a conventional oil-pumped vacuum system, varied markedly from sample to sample. Additionally, a matt black form of carbon was sometimes observed at the edge of deposits when masks were used to define the boundary of growth. A fuller account of this work will be published elsewhere.

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The role of hydrogen sulphide in environmental transport of mercury

HYDROGEN SULPHIDE may be evolved in anoxic sulphur-containing river or lake sediments¹. The effect of the presence of sulphide on the availability of inorganic mercury for biological methylation has been considered previously. Essentially mercury in the form of Hg(0) or Hg(II) may be converted into mercuric sulphide in anoxic sediments². It might seem that this very insoluble material would not, in practice, be available for methylation and that sulphide formation would act as a method of removing mercury from availability to the general environment (that is, it would act as a mercury sink). However in a complex natural system (aquarium sediment) it has been shown that HgS may, in fact, be methylated in the sediments although to produce quantities of methyl mercury substantially less than those produced using a more soluble mercury substrate². Therefore, although H₂S in a sediment would be expected to reduce the rate of mercury methylation considerably by the formation of HgS, it would not be expected to prevent formation of methyl mercury entirely. The effect of H₂S on methyl mercury itself has also been investigated using a purely inorganic model³. An aqueous solution of methyl mercuric chloride (1 mg dm⁻³) loses methyl mercury steadily over 3 days after initial exposure to H₂S. A volatile product was evolved into the space above the solution. This product was trapped in a sodium carbonate-disodium phosphate solution, extracted with toluene and shown to co-migrate with methyl mercury on thin layer plates of silica gel. It was concluded that the evolved product of the reaction system was a volatile organo-sulphur derivative of mercury that reverted to methyl mercury on extraction. These observations suggest that reaction between methyl mercury and H₂S generated in sediments reduces the amount of methyl mercury available for accumulation by fish and shellfish and represents a

powerful mechanism adding to the general mobility of mercury compounds in the environment. It is therefore important to identify the unknown mercury compound evolved from the system described above. We have now investigated the inter-action of H₂S with River Mersey (England) inter-tidal estuary sediment samples in order to determine its effect on methyl mercury levels in real systems. We have also investigated similar reactions in a model system, leading to the identification of the previously unknown volatile mercury species responsible for the transport of mercury in the work of Rowland *et al.*³

Our experiments with the model system were similar to those described previously³, but we used a gas chromatographic technique rather than radiochemical labelling to estimate methyl mercury losses. Aqueous solutions of CH₃HgCl (concentration about 1 mg dm⁻³) were placed in screw top bottles fitted with silicone rubber septa. H₂S or N₂ (for control samples) was passed through the solutions for 2 min. At intervals solution samples were extracted by syringe from each bottle and analysed for methyl mercury as follows.

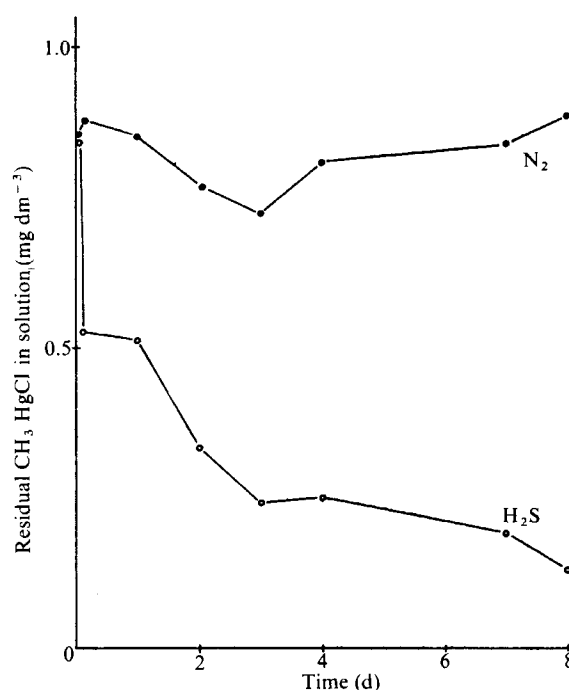


Fig. 1 Volatilisation of CH₃HgCl from aqueous solution by H₂S. Initial concentration of solutions was 1 mg dm⁻³. There was an immediate rapid decline to 0.85 mg dm⁻³ for both solutions owing to adsorption on vessel surfaces. This initial decline over the first few minutes of the experiment has not been included in the graph. Temperature, 18°C.

An aliquot of 0.1 cm³ of each solution was placed in a tube and N₂ gas passed over the solution for 30 s to remove excess H₂S. (CH₃HgCl is not affected by this procedure.) The sample solution was then shaken with 1 cm³ benzene (GLC grade) and 0.1 cm³ CuSO₄ solution (10%) for 2 min. The benzene layer was removed and the methyl mercury content determined by electron-capture gas chromatography as described previously⁴. We found that methyl mercury was steadily lost from the original solution and a typical example from an experiment of this kind is shown in Fig. 1. These results are not incompatible with those of Rowland *et al.*³ although our experiments were conducted over a rather longer period.

As the gas chromatographic technique used was probably specific for monomethyl mercury, an attempt was made to identify any other species in the solution using ¹H NMR spectroscopy. The NMR spectrometer used (Jeol C60-HL) could detect methyl mercury in concentrations of ≥0.5%. Because of solubility difficulties with the chloride we carried out NMR experiments using methyl mercuric acetate at a concentration of

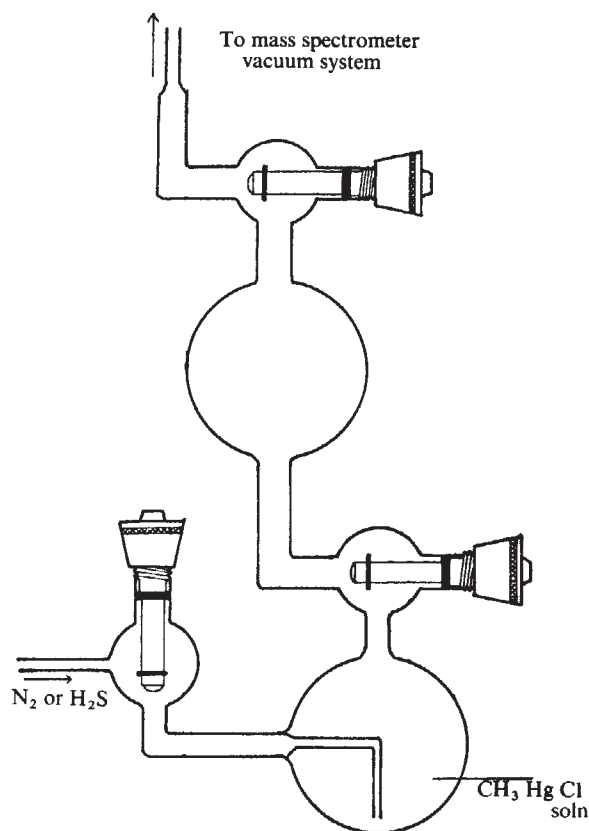


Fig. 2 Two chamber vacuum vessel. Apparatus designed for connection to mass spectrometer vacuum line. (Taps used: glass/PTFE—J. Young, London.)

1% in D_2O . NMR spectra of $CH_3HgOCOCH_3$ in D_2O show three principal peaks for methyl mercury, acetate-methyl and residual water at 9.0, 8.0 and 4.3 τ values respectively. For the H_2S experiment, 0.25 cm^3 of 2% $CH_3HgOCOCH_3$ solution in D_2O was mixed with 0.25 cm^3 of saturated H_2S solution in D_2O in an NMR tube (a white precipitate formed immediately). The tube was then sealed and a spectrum recorded. The NMR spectrum showed peaks only for acetate-methyl and H_2O . The H_2S peak did not appear owing to proton exchange with D_2O . The absence of the methyl mercury peak shows that the formation of the white precipitate has reduced the methyl mercury concentration in the solution to below that detectable by the NMR technique.

The control (no H_2S) and the experimental sealed NMR tubes were re-examined several times over a 14-day period. No changes in the spectra were seen over this period. The white precipitate, however, did change colour slowly from white to black.

The initial white compound was prepared, separately, from H_2S solution and $CH_3HgOCOCH_3$. After recrystallisation from ethanol, the melting point was found to be 142°C at which temperature the compound decomposed to a black solid. A mass spectrum was run of the white compound whose highest m/e values and characteristic mercury isotope pattern (see for example ref. 5) and distribution demonstrated it to be $(CH_3Hg)_2S$. The melting point is consistent with the literature melting point (143°C) for $(CH_3Hg)_2S$ (ref. 6).

It seems then that loss of methyl mercury from aqueous solution on exposure to H_2S is caused initially by formation of $(CH_3Hg)_2S$. The solutions used by Rowland *et al.* and in our GLC experiments were much more dilute than the solutions used for NMR, and this might account for a slower appearance of $(CH_3Hg)_2S$ in our GLC work. The subsequent change of

white $(CH_3Hg)_2S$ to a black material represents a further reaction that may account for the actual volatilisation and loss of mercury from the system.

An experiment was devised to try to identify the volatile product(s) by mass spectrometry. A two-chamber vessel (Fig. 2) was constructed such that the aqueous phase reaction between CH_3HgCl and H_2S could be carried out with subsequent separation of any volatile products. Twenty-five cm^3 of aqueous CH_3HgCl (1 g dm^{-3}) were placed in the lower section and N_2 (control experiment) or H_2S was passed through the solution for 2 min. With H_2S a white precipitate formed immediately. After 3 days in the closed system the upper chamber was evacuated and any volatiles present in the lower chamber were allowed to pass into this upper section. The apparatus was connected to a vacuum line system attached to a mass spectrometer (V.G. Micromass 16B). The contents of the upper vessel were trapped out using liquid nitrogen in a section of the vacuum system. After initial warming excess H_2S was pumped away and the trap contents were allowed to enter the mass spectrometer. The characteristic mass spectrum of dimethyl mercury was observed. The compound was identified by the characteristic isotope pattern observed for mercury⁵ surrounding m/e 232, 217 and

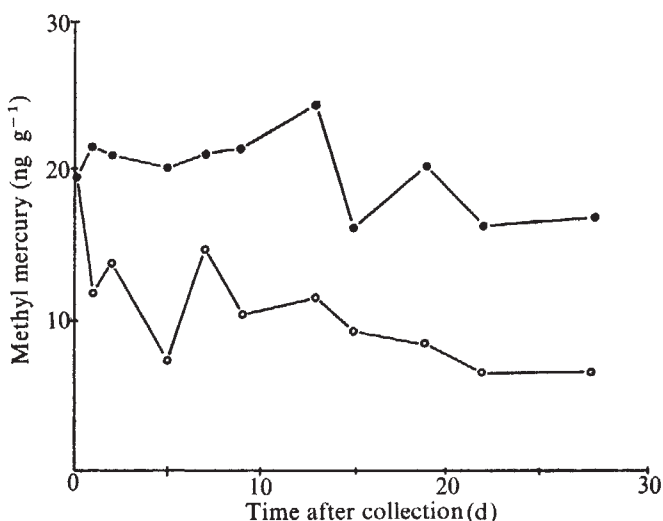


Fig. 3 Analysis of sediment samples (Hale Point, Mersey Estuary) for methyl mercury. Samples stored at room temperature (18°C). ●, Untreated; ○, H_2S treated samples.

202 corresponding to $(CH_3)_2Hg^+$, CH_3Hg^+ and Hg^+ . The control sample did not evolve observable amounts of CH_3HgCl into the vapour phase and hence we conclude that our observed CH_3Hg^+ and Hg^+ peaks were caused by breakdown of $(CH_3)_2Hg$ not evolution of CH_3HgCl . In any case we observe no peaks corresponding to m/e 254 and 252 for CH_3HgCl , nor any $HgCl^+$ species at 239 and 237. This evidence suggested that only $(CH_3)_2Hg$ had been evolved. We could find no evidence for CH_4 or any mercury sulphur species. Comparison with standard mass spectra of known samples of $(CH_3)_2Hg$ and CH_3HgCl reinforced these conclusions. We could find no evidence for the presence of suitable mass ratio peaks for compounds such as CH_3HgSCH_3 and CH_3HgSH . The H_2S experiment was repeated using a 1 mg dm^{-3} solution of CH_3HgCl but this was too low a concentration for detection in our apparatus.

The results of this series of experiments suggest that Rowland *et al.*³ observed the evolution of volatile $(CH_3)_2Hg$ from the reaction between H_2S and CH_3HgCl . $(CH_3Hg)_2S$ was apparently formed as an intermediate in the reaction. This compound gradually decomposes to black β - HgS and $(CH_3)_2Hg$. This is in accord with the known chemistry of this species^{7,8}.

Our results for CH_3HgCl levels in solution differ in small detail from those of Rowland *et al.* who measured total CH_3Hg species remaining in solution, whereas our technique measures water soluble CH_3Hg species remaining in solution. This would include CH_3HgCl and possibly, to a small extent, $(\text{CH}_3\text{Hg})_2\text{S}$. In our experiments a precipitate of $(\text{CH}_3\text{Hg})_2\text{S}$ was observed. Rowland *et al.* do not mention this and it is likely that at the higher temperatures they used (37°C) a precipitate was either not present or present only to a very small extent. Our experiments were carried out at 18°C .

The rapid early loss of methyl mercury found in our experiments is probably due to initial rapid formation of some $(\text{CH}_3\text{Hg})_2\text{S}$ to an equilibrium level, followed by a slower decay of the sulphide to dimethyl mercury which is lost from solution.

The question arises as to how much significance these reactions have for environmental situations. In our experiments on the analysis of estuarine sediments for methyl mercury we have observed changes in methyl mercury levels over a number of days following sampling⁴. Changes in the redox conditions of the sediment following sampling were suspected as a possible cause of this effect. In a series of such experiments we passed H_2S through a container of freshly-sampled sediment; the sediment was later sub-divided into a series of bottles. Single bottles were analysed for methyl mercury over 28 days and compared with a control set which was not exposed to H_2S .

These experiments have demonstrated that diffusion of H_2S in a sediment significantly reduces the level of methyl mercury in that sample relative to a control (Fig. 3.). We have also been able to show that dimethyl mercury is evolved into the gaseous phase from a mercury-containing sediment which has been treated with H_2S . A control sediment not exposed to H_2S did not evolve $(\text{CH}_3)_2\text{Hg}$ in detectable quantities. For this latter experiment we were forced to raise the concentration of methyl mercury to $100\text{ }\mu\text{g g}^{-1}$ by 'spiking' with CH_3HgCl . At natural concentrations of methyl mercury, of the order of 15 ng g^{-1} , we could not detect $(\text{CH}_3)_2\text{Hg}$ in control or H_2S exposed samples.

Our results suggest that monomethyl mercury species in sediments may be mobilised into the atmosphere as $(\text{CH}_3)_2\text{Hg}$ if H_2S is generated in the sediments. This points to a natural mechanism for the removal of mono-methyl mercury from situations where it may be incorporated into fish. In positively identifying $(\text{CH}_3)_2\text{Hg}$ as the volatile species another route in the general mercury cycle has been demonstrated. In view of the potential generation of further monomethyl mercury species from the large 'reservoir' of inorganic mercury that accompanies monomethyl mercury in sediments⁹, there is the possibility of volatilisation of larger amounts of mercury than the equilibrium amounts of methyl mercury present.

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Reevaluation of the role of dimethyl sulphide in the sulphur budget

THE possible existence of an oceanic source of atmospheric SO_2 has recently been suggested for the following reasons. First, to account for the observed¹ SO_2 background concentration of $0.1 \times 10^{-6}\text{ g m}^{-3}$ of air, even very far from any continental source and in areas as remote as the Antarctic ocean—despite a residence time for SO_2 in the marine atmosphere of $\sim 0.7\text{ d}$ (refs 2, 3). Second, because another major source such as the ocean is necessary to complete the global atmospheric sulphur budget^{4–7}. Because of the basic pH of seawater, SO_2 cannot be emanated directly from the oceans. According to Lovelock *et al.*⁸, the dimethyl sulphide (DMS) produced in the sea by many living systems, could be evolved in a first step into the atmosphere and subsequently oxidised to SO_2 . These authors observed a mean DMS concentration of the order of $12 \times 10^{-9}\text{ g l}^{-1}$ in Atlantic ocean water. We have developed an improved method for the measurement of DMS in seawater and have used this technique on several recent oceanographic research cruises. Our results suggest that the oceans could contribute over 30% of the amount of sulphur required to balance the sulphur budget.

DMS was first extracted from the seawater using carbon tetrachloride, then by 5% aqueous solution of mercuric chloride as a Hg^{2+} -DMS complex which remains stable for more than 6 months. In the laboratory, DMS was released by 6 M HCl, redissolved in propan-2-ol at -35°C and finally measured by gas chromatography with a flame photometric detector. The limit of detection is $5 \times 10^{-11}\text{ g}$ of DMS. With the usual yields, this figure corresponds to a limit of $5 \times 10^{-9}\text{ g l}^{-1}$ for a 15-l sample of seawater.

A preliminary list of DMS concentrations for the Atlantic and Indian Ocean waters is shown in Table 1. The lowest values found agree well with the results of Lovelock *et al.*⁸. The highest concentrations observed (samples 1, 4 and 12) characterise areas with presence of algae (principally of *Fucus* and *Ascomyllum* with *Polysiphonia Lanosa*). Sample 2 was taken in water highly polluted by oil, 1 week after the oil spill of the Amoco Cadiz.

We determined levels of DMS in the microlayer and the concentration distribution with depth in the Mediterranean. To the best of our knowledge, this is the first report of such measurements. Surface microlayer samples (about $400\text{ }\mu\text{m}$) were obtained by using the Garrett screen⁹. The DMS concentrations found for these samples are given in Table 2.

As a general rule, the values observed in the Mediterranean are significantly higher than in the Atlantic. The concentrations

Table 1 Concentration of DMS in Atlantic and Indian Ocean seawater

Sample no.	Location	Date of sampling	Concentration (10^{-9} g l^{-1})
1	Northern coast of Brittany	February 1978	38 ± 8
2	Portsall (Brittany)	March 1978	19 ± 4
3	Roadstead of Brest	March 1978	7 ± 1
4	Ré island	May 1978	23 ± 5
5	Ré island	May 1978	11 ± 2
6	Ré island	May 1978	15 ± 3
7	Oleron island	May 1978	11 ± 2
8	Oleron island	May 1978	13 ± 3
9	Oleron island	May 1978	13 ± 3
10	Oleron island	May 1978	24 ± 5
11	Guinea Gulf ($2^\circ 35'\text{S}$ – $3^\circ 02'\text{E}$)	June 1977	14 ± 3
12	Amsterdam island ($37^\circ 47' 30''\text{S}$ – $77^\circ 32'\text{E}$)	March 1977	34 ± 7

Table 2 Concentration of DMS in Mediterranean seawater

Sample no.	Date	Location	Concentration (10^{-9} g l^{-1})		Sea state and temperature of surface water
			Microlayer	Subsurface	
13	28 June 1977 16 h–18 h	7°30' E 41°20' N	1,500 ± 300	580 ± 116	Calm
14	29 June 1977 15 h–16 h	7°30' E	800 ± 160	150 ± 30	Calm
15	5 April 1978 14 h 30–15 h 30	7°48' E 43°17' N	70 ± 14	60 ± 12	Calm 13 °C
16	6 April 1978 10 h 30	7°23' E 43°38' N		60 ± 12	Rough 13 °C
17	7 April 1978 10 h	7°24'30" E 43°36'30" N		51 ± 10	Rough 13 °C
18	26 April 1978 15 h 30	43°22' 7°40' E		17 ± 3	Rough 14.70 °C
19	27 April 1978 10 h	43°36' N 7°25' E		15 ± 3	Rough 14.80 °C
20	28 April 1978 13 h	43°22' N 7°44' E		11 ± 2	Rough 13.80 °C

measured in samples 13 and 14 seem to be anomalously high. Possible contamination during the sampling can be ruled out, however, because of the strict precautions taken (for instance, the water was sampled from a small dinghy 1 km forward of the ship). The microlayer is more concentrated in DMS than the subsurface water, with ratios of microlayer and subsurface

levels of between 1.2 and 5.3. Similar ratios (~ 3) have been observed for particulate organic carbon and nitrogen in the microlayer^{10–13}.

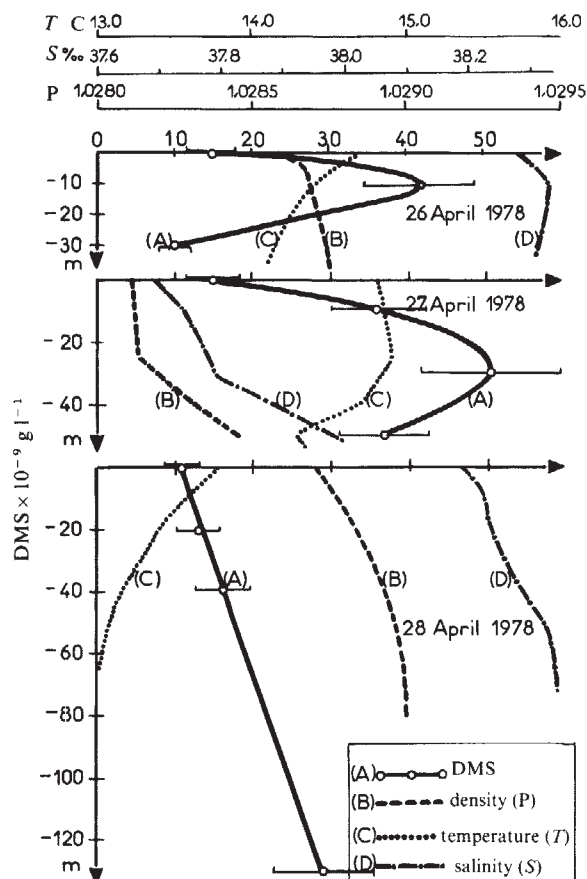
The DMS depth profiles obtained are shown in Fig. 1 together with those of temperature, salinity and density of the seawater. These profiles show the existence of high DMS concentration layers under the sea surface. The maximum DMS concentrations seem to be connected with a sharp variation in the gradient of density and therefore to a dynamically stable water layer. A similar observation is usual in the case of marine particulate matter.¹⁴

From these preliminary studies, we can draw several conclusions. First, a very large range of DMS concentrations is observed in the superficial seawater, the figures previously obtained by Lovelock⁸ appearing to be a lower limit rather than typical of the average oceanic value. Second, the DMS concentration also varies quite rapidly with depth: factors of 3 have been observed, as in Fig. 1, between the surface and the depth of the maximum concentration (10 and 30 m). These maximum DMS concentration layers seem to be able to move up, and to reach the surface, which could explain the existence of sometimes very high surface DMS concentrations. Both of these observations suggest that the mean oceanic DMS concentration should be of the order of $30 \times 10^{-9} \text{ g l}^{-1}$ instead of $12 \times 10^{-9} \text{ g l}^{-1}$ indicated by Lovelock⁸. Third, from the knowledge of the oceanic content of DMS, it should be possible to evaluate the flux of this sulphur compound to the atmosphere. Liss and Slater¹⁵ have suggested a method of calculation based on Fick's law of molecular diffusion and a Henry's law constant of 0.3:

$$F = K_1(C_g/0.3 - C_1)$$

where F is the DMS flux ($\text{g cm}^{-2} \text{ h}^{-1}$), K_1 the exchange constant equal to 19.2 cm h^{-1} , C_g and C_1 respectively the DMS concentration in the air (negligible¹⁶) and in the seawater immediately under the molecular diffusion layer, that is here the microlayer concentration.

According to the present work, $C_1 > 90 \times 10^{-9} \text{ g l}^{-1}$ which is 3 times the mean subsurface concentration, since the sampling method of the microlayer partially mixes the superficial film with the subsurface water. This formula gives for the oceans a flux of at least $2.7 \times 10^{13} \text{ g yr}^{-1}$ of sulphur. Such a flux could represent more than 30% of the amount needed to balance the sulphur budget.

Fig. 1 DMS depth profile.

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Derivation of K-rich ultramafic magmas from a peridotitic mantle source

THE genesis of a wide spectrum of basaltic magmas by partial melting of a peridotitic upper mantle with small amounts of H₂O as its only volatile component is well established^{1–3}, as is the role of CO₂ in magma genesis in the upper mantle^{4,5}. However, the origin of K-rich mafic to ultramafic magmas has not been satisfactorily explained by mechanisms involving partial melting of commonly accepted upper mantle sources. We report here experiments on an olivine ugandite from Bufumbira, south-west Uganda⁶, in conditions of H₂O and H₂O–CO₂ mixtures ($x_{\text{CO}_2} \sim 0.75$) up to 40 kbar, and at an $f_{\text{O}_2} \approx \text{Ni–NiO}$ buffer⁷. These are the first experiments reported on a lava of the ultramafic alkaline type with K₂O > Na₂O using both H₂O and CO₂ as volatiles and showing that K-rich ultramafic magmas can be derived by partial melting of a 'normal' peridotitic mantle containing both H₂O and CO₂. Olivine ugandite is believed to be the most primitive lava occurring in this K-rich province^{6,8–10} and may reasonably be considered as a model composition for K-rich ultramafic magmas generally.

Magmas with increasing degrees of undersaturation can be produced only by progressively decreased amounts of partial melting of a peridotitic upper mantle if H₂O is the only volatile component present³; olivine nephelinites or olivine basanites represent the most undersaturated types¹¹. Experiments up to 40 kbar on an olivine melilitite^{7,11,12} indicate that partial melting of an upper mantle source containing H₂O alone cannot produce such a magma, whereas partial melting of an upper mantle peridotite containing both H₂O and CO₂ ($x_{\text{CO}_2} \sim 0.75$) provides a satisfactory mechanism. Experiments on such magmas using both H₂O and CO₂ as volatile components have only involved compositions with Na₂O $\geq$ K₂O. A high pressure study¹³ of a very K₂O-rich lava shows that such lavas cannot be produced by partial melting of a peridotitic mantle in which H₂O is the only volatile component.

Figure 1a shows the results of experiments in anhydrous, 5%, 15% and water-saturated conditions. The absence of orthopyroxene and garnet and the dominance of olivine and clinopyroxene at, or near, liquidus temperatures precludes the possibility of this magma being derived by partial melting of a peridotitic mantle source containing only H₂O. In contrast to studies of a composition richer in K₂O (ref. 13), phlogopite in the olivine ugandite does not occur in near-liquidus conditions (Fig. 1b), indicating that mechanisms of differentiation involving early fractionation of phlogopite¹³ are untenable.

Figure 1c shows phase relations with $x_{\text{CO}_2} \approx 0.75$ representing 27.5% weight CO₂ and 3.8% weight H₂O (equivalent to the 15% weight H₂O conditions of Fig. 1b). In these conditions, orthopyroxene, clinopyroxene and spinel are the near-liquidus

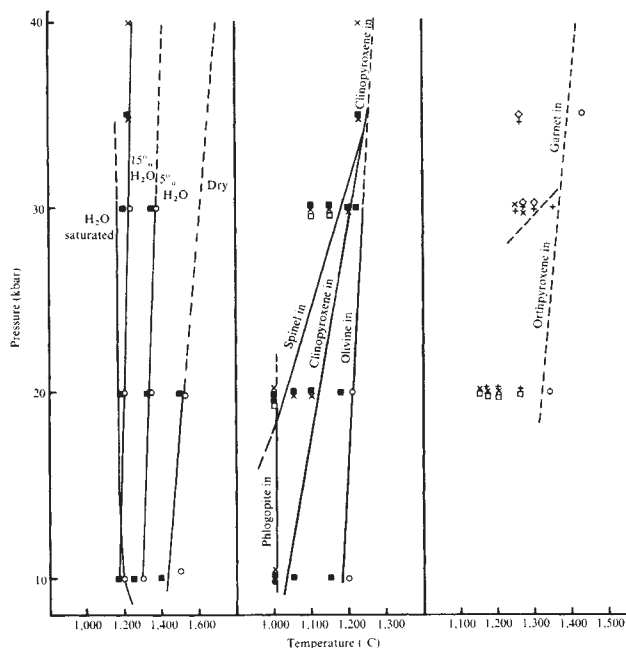


Fig. 1 *P–T* projection of olivine ugandite composition. *a*, Liquidus relations in anhydrous, 5% H₂O, 15% H₂O and H₂O-saturated conditions. *b*, Liquidus and subliquidus relations at 15% H₂O. *c*, Liquidus and subliquidus relations with H₂O and CO₂ at $x_{\text{CO}_2} \approx 0.75$ (mol %). ○, Above liquidus; ●, olivine; ×, clinopyroxene; +, orthopyroxene; ●, phlogopite; □, spinel; ◇, garnet.

phases at 20 kbar and orthopyroxene is the near-liquidus phase at 30 kbar, closely followed by garnet and clinopyroxene. Comparison of these results with those for an olivine melilitite^{7,11,12} indicates that the olivine ugandite could be produced by very small amounts of partial melting (<5%) of a peridotitic mantle source containing both H₂O and CO₂.

Calculations using the microprobe analyses of the minerals crystallised in our experiments indicate that it is highly unlikely that mechanisms involving fractionation of garnet and/or orthopyroxene can develop a K-enrichment trend from parental magmas of olivine ugandite compositions. However, calculations using data on the minerals present in the runs done with 15% H₂O as the only volatile component (Fig. 1b) show that sequential fractionation of olivine, olivine + clinopyroxene, and olivine + clinopyroxene + phlogopite between about 20 kbar and 1,200 °C to about 10 kbar and 1,000 °C can produce successive liquids with compositions closely corresponding to the olivine ugandite–ugandite–olivine leucite–leucite series of the Bufumbira region⁶. Such liquids show an enrichment in K relative to Na (as 100 K/(K + Na)) from 41 in the olivine ugandite to 55 in the leucite, with a concomitant decrease in 100 Mg/(Mg + Fe²⁺) from 81 to 46.

On the basis of these experiments using olivine ugandite as a model parental composition, we propose that such magmas may be derived by small amounts of partial melting from a 'normal' (non K-enriched) peridotitic mantle source containing H_2O and CO_2 as essential constituents at ≥ 30 kbar and $\geq 1,350^\circ C$. In conditions of crustal warping due to rifting¹⁴, this magma may rise and its temperature decrease, producing a decrease in the solubility of CO_2 relative to H_2O (ref. 15) which suppresses crystallisation of garnet and orthopyroxene. At approximately 20 kbar and $1,200^\circ C$, olivine begins to fractionate, followed by olivine + clinopyroxene at lower pressures and temperatures (Fig. 1b). Residual liquids are depleted in MgO and enriched in TiO_2 , Al_2O_3 , CaO, Na_2O and K_2O . With further decrease in pressure and temperature, this residual liquid incorporates phlogopite which has previously crystallised from one of the liquids derived from the olivine ugandite or, less likely, from the olivine ugandite itself. This phlogopite may crystallise at ≈ 20 kbar (Fig. 1b) and rise by flotation to shallower depths equivalent to pressures of 10–20 kbar. With the addition of phlogopite and further fractionation of olivine and clinopyroxene, liquids substantially enriched in K_2O relative to Na_2O can be evolved at 10–20 kbar and $\geq 1,000^\circ C$. This mechanism of K-enrichment for lavas with $K_2O > Na_2O$ differs from those previously proposed^{6,8–10,16} in that the phlogopite is derived from the parental magma or its derivatives and is not a xenocrystic phase.

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Late Precambrian microfossils from Brioverian cherts and limestones of Brittany and Normandy, France

THE discovery of assemblages of unicellular, colonial and multiunit (endosporangium-like) algal microfossils in thin sections and in macerations of carbonaceous cherts and dolomitic limestones from the late Precambrian Brioverian 'Series' of northwestern France is reported here. These assemblages represent new links in the now rapidly expanding known record of Precambrian life. They are of interest because of (1) their similarity to microbiotas of comparable age reported from Canada and Alaska, a similarity suggesting that such assemblages could prove useful for global biostratigraphic correlation of late Precambrian strata; (2) their presence in partially metamorphosed sediments, an occurrence indicating that Precambrian organic-walled microfossils may not be as susceptible

to metamorphic alteration as has widely been assumed; (3) one of the assemblages is preserved in carbonates, a facies previously little studied by Precambrian micropaleontologists.

Brioverian rocks are of demonstrably Precambrian age: they underlie lavas having a radiometric age of about 550 Myr (ref. 1); in Normandy, they unconformably underlie sediments containing a lower Cambrian fauna, and they are older than intrusive granites dated at about 580–620 Myr (ref. 2). The sequence is apparently substantially younger than 2,000 Myr, the approximate age of the underlying gneissic basement^{3,4}.

In Brittany and Normandy, Brioverian rocks can be subdivided into two age-groups: (1) a lower, metamorphosed sequence which in its upper portions contains metasediments (including black cherts locally known as 'phtanites') and which is older than 695 Myr, the age of plutonic massifs intrusive into the sequence^{2,3}; (2) a younger, unconformably overlying sequence which at its base locally contains volcanic material dated at about 640 Myr (ref. 4) and which is comprised largely of laminated shales and siltstones containing lenses of sandy dolomitic limestone. Three categories of microfossils (types I–III, below) were detected in lower Brioverian black cherts from the Pont–Mazet Quarry, near Quibou (between Coutances and St-Lô), Normandy, and from Le Pelikan (near Lamballe), Brittany; a fourth category (type IV) has been discovered in upper Brioverian dolomitic limestones from Vern-sur-Seiche (near Rennes), Brittany (Fig. 1).

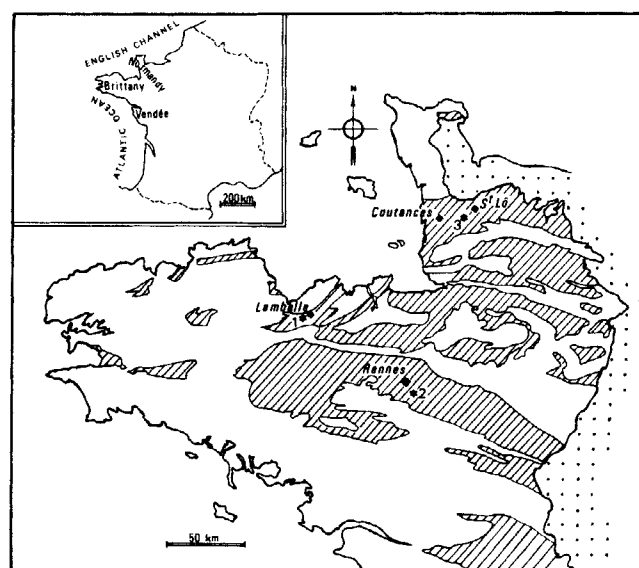


Fig. 1 Sketch map showing distribution of late Precambrian (cross-hatched) and Mesozoic–Cainozoic (stippled) sediments in Brittany and Normandy and locations of fossiliferous lower Brioverian black cherts (1, Le Pelikan, near Lamballe; 3, Pont–Mazet Quarry, near Quibou) and of upper Brioverian dolomitic limestones (2, Vern-sur-Seiche, near Rennes).

Like previously described microfossils from other fine-grained, carbonaceous Precambrian cherts^{5,6}, those of the lower Brioverian cherts have been preserved by siliceous permineralisation in a three-dimensional, structurally little altered condition (Fig. 2a–q). Organic matter comprising these fossils ranges in colour from light-grey to dark-grey or nearly black; like the particulate kerogen that is dispersed throughout these cherts, the carbonaceous microfossils were evidently heated, geochemically altered and 'coalified' during metamorphism of the sedimentary sequence. Microfossils detected in the upper Brioverian dolomitic limestones (Fig. 2r–v) are composed of light- to dark-brown organic matter, are permineralised in carbonate, rather than in silica, and in some cases seem to have been physically disrupted during compaction and diagenesis of the surrounding carbonate matrix (for example, Fig. 2v). These

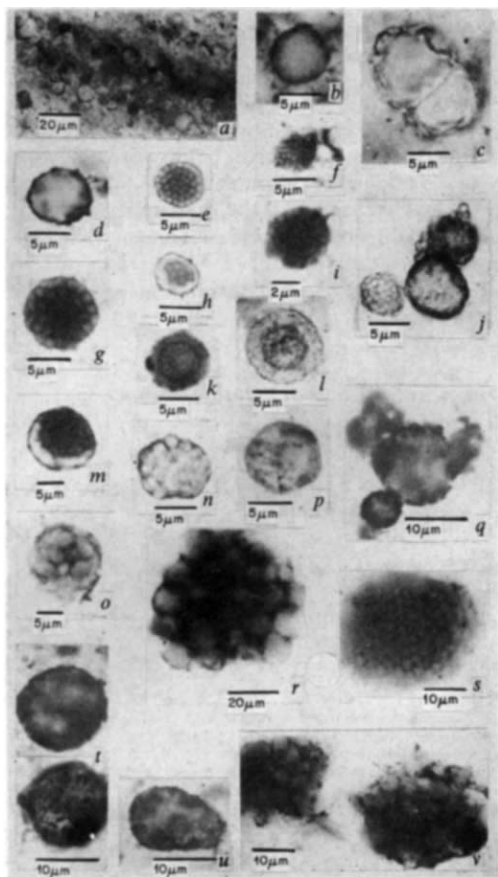


Fig. 2 Late Precambrian, organically preserved microfossils in thin sections (*a-c, f, i, j, l, p, r, s, v*) and in macerations (*d, e, g, h, k, m-o, q, t, u*) of lower Brioverian black cherts from Pont-Mazet, Normandy (*a-o*) and from Le Pelikan, Brittany (*p, q*), and of upper Brioverian dolomitic limestones from Vern-sur-Seiche, Brittany (*r-v*). Type I microfossils occur singly (*b, d, p, q*), in cell-pairs (*c*) or in irregular colonies (*a*); type II microfossils contain many spore-like coccoids (*e-m*); type III microfossils contain fewer, somewhat larger, spore-like bodies (*n, o*); type IV microfossils occur singly (*t, u*) or in globular colonies (*r, s, v*).

observations, together with studies of the textural relationships between these fossils and their encompassing mineral matrices and the fact that each of the four types of microfossils occurs abundantly (as demonstrated by studies both of acid-resistant macerations and of petrographic thin sections), establish that the Brioverian unicells and colonies are *bona fide* Precambrian fossils: they are indigenous to sediments of established Precambrian age and they were deposited syngenetically with Precambrian sedimentation.

Type I microfossils are spheroidal, thin-walled, unornamented unicells 2–15 μm in diameter (mean diameter = 5.6 μm , $n = 993$; Fig. 3*a*), that occur singly (Fig. 2*b, d, p*), in partially divided cell-pairs (Fig. 2*c*), or in mucilage-ensheathed, irregular colonies composed of a few to hundreds (Fig. 2*a*) of closely packed cells. Such cells comprise about 60% of microfossils detected in the lower Brioverian cherts.

Type II microfossils are spheroidal, noncolonial, multiunit cells, 2–12 μm in diameter (mean diameter = 4.9 μm , $n = 472$; Fig. 3*a*), that contain many spore-like, coccoidal bodies. These internal, closely packed coccoids, which in well-preserved specimens (for example, Fig. 2*e, g*) form three to four contiguous layers or 'shells' concentric about the centre of the spheroids, range in diameter from <0.5 to about 2 μm . A thin, unornamented cell wall can be discerned in those spheroids in which the spore-like coccoids only partially fill the enclosing cell (Fig. 2*h, m*), a condition resulting from degradation of the internal coccoids or, as evidenced by the common occurrence of

solitary, μm -sized, spore-like bodies in the cherts, from the release of such coccoids into the surrounding medium before preservation. Type II microfossils constitute nearly 40% of all forms detected in the lower Brioverian cherts.

Type III microfossils are noncolonial, multiunit, spheroidal cells 7–10 μm in diameter, that enclose two or three concentric 'shells' of closely packed, spore-like bodies (Fig. 2*n, o*). These bodies range in diameter from <2 to ~4 μm ; such cells thus contain fewer, but appreciably larger, coccoidal bodies than those contained in type II microfossils. Type III microfossils comprise only a small portion (<1%) of either of the two assemblages detected in the Brioverian cherts. Cells of this category, and of type II microfossils, are very similar in size to the type I microfossils described above (Fig. 3*a*). Indeed, except for the occurrence of colonality (exhibited only by type I microfossils) and of distinctive, internal, spore-like bodies (characteristic of types II and III), members of the three categories of microfossils are virtually indistinguishable.

Thus, although additional morphotypes may also be represented, the lower Brioverian 'cherts' from the Pont-Mazet Quarry and Le Pelikan contain at least two morphologically distinct types of unicellular fossils: colonies and (at least in part) solitary type I microfossils; and type II + (?) type III + (in part ?) solitary type I microfossils. These Brioverian assemblages bear only limited resemblance to microfloras previously described from late Precambrian cherts of western Europe⁷, for although simple 'type I-like' unicells (for example, *Paleocryptidium cayeuxi*) have been detected in Brioverian black cherts at Lamballe, Brittany⁹, and in late Proterozoic cherts of Bohemia¹⁰, they have not been found in association with the distinctive multiunit microfossils described here. In contrast, however, the Pont-Mazet and Le Pelikan assemblages seem strikingly similar to microfloras recently reported by Moorman from late Precambrian sediments of North America^{11,12}, microbiotas regarded by her as being composed solely of growth stages of a single taxon, *Sphaerocongregus variabilis*, a coccoid blue-green alga somewhat similar to the modern pleurocapsalean *Pleurocapsa fuliginosa*. According to Moorman¹¹, solitary or colonial unicells (as in our type I microfossils) represent vegetative growth stages of *S. variabilis* multiunit cells containing many small, spore-like bodies (as in our type II microfossils) are reproductive endospores, and multiunit cells that contain fewer, larger, coccoids (as in our type III microfossils) are sporangia containing 'nannocytes'. Fossils of the Pont-Mazet Quarry, Le Pelikan and North American assemblages seem virtually identical in morphology; thus, although the Brioverian material does not permit us to substantiate all phases of the "hypothetical growth cycle" proposed by Moorman for *S. variabilis*¹¹ (in particular, we find no evidence of the multiserial,

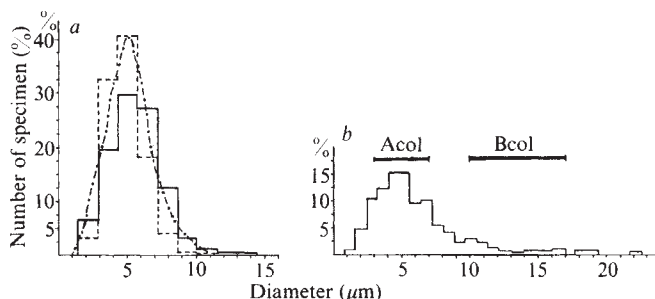


Fig. 3 *a*, Size distribution of colonial type I microfossils (---, $n = 260$, measured in thin sections), of solitary type I microfossils (—, $n = 733$, measured in macerations), and of type II microfossils (···, $n = 472$, measured in macerations) from lower Brioverian black cherts of Normandy and Brittany. *b*, Size distribution of type IV microfossils from upper Brioverian limestones of Vern-sur-Seiche, Brittany; histogram shows sizes of solitary cells measured in macerations ($n = 386$); Acol ($n = 29$) and Bcol ($n = 23$) show size ranges for cells in two well-preserved colonies, measured in thin sections.

vegetative, filament-like structures she depicts), these newly discovered assemblages support several of her interpretations. Specifically, it seems increasingly plausible (1) that the multiunit cells may represent reproductive stages, and that the solitary and colonial cells may be vegetative stages of a single taxon; (2) that this taxon is probably of blue-green algal affinity (although its referral to the Pleurocapsales remains in question, as endosporulation also occurs among chroococcalean and chamaesiphonaeal cyanophytes); and (3) that *S. variabilis* was chiefly planktonic, rather than benthonic, in habit (an interpretation supported in both the North American and French material by, among other lines of evidence, the more-or-less random distribution of the fossils within the sediments). In addition, the Brioverian specimens of *Sphaerocongregus*, in which the cells are preserved as organic residues rather than as 'pyritic replacements'¹², confirm that the multiunit cells are authentic microfossils rather than the nonbiological pyrite framboids to which they bear superficial resemblance^{13,14}.

In North America, *S. variabilis* has been detected in shales of the 850–570 Myr-old Hector Formation of the Windermere Supergroup of southwestern Alberta, Canada¹¹, and in carbonaceous shales and mudstones of approximately the same age from the Tindir Group of east-central Alaska, USA¹². Multiunit microfossils similar to the types II and III microfossils described here have also been reported from sediments of the Soviet Union (small-celled species of *Symplastosphaeridium* Timofeev = *Microconcentrica* Naumova) in which they reportedly range in age from the mid-part of the Middle Riphean (about 1,100 Myr in age) into the Late Cambrian¹⁵. Based on these known distributions, and in view of stratigraphical and geochronological data available for the Brioverian sequence, it seems reasonable to suggest that the fossiliferous lower Brioverian phanites at Pont-Mazet and Le Pelikan are probably Middle to Late Riphean (1,100–695 Myr) in age.

Type IV microfossils (Fig. 2*r–v*), detected in upper Brioverian (Vendian age) sandy dolomitic limestones from Vern-sur-Seiche (Fig. 1), are spheroidal, relatively thick-walled (Fig. 2*r, t*), unornamented unicells, 1–>20 µm in diameter (mean diameter = 5.9 µm, *n* = 386; Fig. 3*b*), occurring singly (Fig. 2*t, u*) or in globular colonies composed of a few to many cells (Fig. 2*r, s, v*). Colonies range from <30 to >70 µm in diameter and have a measured abundance of about 50 colonies per cm³ of rock; they most commonly occur within (and tend to be orientated parallel to) fine, organic, bedding-plane laminations, a distribution suggesting that they were predominantly benthonic in habit. In size, shape and colonial organisation, type IV fossils bear considerable resemblance to modern, coccoid (for example, chroococcalean) blue-green algae, and the broad size range exhibited by the total assemblage, in comparison with the rather uniform size of member of single colonies (Fig. 2*r, s*; Fig. 3*b*), indicates that several cyanophytic taxa are probably represented. Fossils of this size and organisation have been reported from many Precambrian deposits⁵; the Vern-sur-Seiche assemblage, however, is unusual in that only one¹⁶ other of the nearly four dozen permineralised microbiotas now known from the Precambrian^{5,6} has been discovered in carbonates, the remaining permineralised assemblages occurring exclusively in bedded cherts or in cherty portions of algal stromatolites.

In addition to representing new links in the now rapidly expanding knowledge of Precambrian life, the three microfossil assemblages described here are of interest for the following reasons: (1) two of these assemblages, those occurring in the lower Brioverian phanites, are similar in composition to microfloras of apparently comparable age in North America; taken with other data⁵, these similarities suggest that such assemblages may ultimately provide a reliable basis for global biostratigraphic correlation of Precambrian strata. (2) The occurrence of structurally preserved microorganisms in partially metamorphosed Brioverian cherts seems to indicate that Precambrian organic-walled microfossils may not be as susceptible to metamorphic alteration as has widely been assumed; there is thus good reason for continued palaeobiological and biostratigraphical studies of such facies, especially in western France where many cherts of similar metamorphic grade but of uncertain geological age occur. (3) The occurrence of permineralised, well-preserved microfossils in upper Brioverian limestones suggests that in addition to shales and cherty facies, carbonates represent a productive, but as yet largely untapped, source of new evidence regarding the diversity, evolution and biostratigraphical usefulness of the Precambrian biota.

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Influence of a predator on the optimal foraging behaviour of sticklebacks (*Gasterosteus aculeatus* L.)

ACCORDING to the principle of natural selection, each individual animal is assumed to maximise its inclusive fitness¹. Thus, observed behaviour patterns should result from optimisation processes involving costs and benefits measured in a currency of fitness^{2,3}. Certain foraging strategies have been shown to maximise energy intake per unit time^{3–6}. Maximisation of the rate of energy intake, however, is an optimal strategy only if feeding behaviour does not conflict with other needs, such as the detection of predators. If the foraging animal runs a high risk of being preyed upon, the optimal strategy may be a compromise of both needs. A predator's influence on optimal foraging has been dealt with, so far, only in a hypothetical manner^{3,5}. We have investigated this problem experimentally using three-spined sticklebacks (*Gasterosteus aculeatus*) attacking a swarm of water fleas (*Daphnia magna*). We report here that after exposure to a model of an avian predator the sticklebacks' foraging behaviour changes such that they attack swarm regions of lower density which provide a lower feeding rate but should increase their ability to detect an approaching predator. This is predicted by a model using Pontryagin's principle of maximisation.

Sticklebacks first attacked swarm regions of high prey density, but with decreasing attack readiness (for example, induced by decreasing hunger) showed a preference for less dense regions, directing their last attacks at stragglers⁷. A high swarm density

enables the predator to achieve a high feeding rate because of short inter-prey distances. However, at high prey densities the predator has increased difficulty in aiming at individual prey targets^{8,9} ('confusion effect'). Thus it seems likely that feeding in high swarm densities reduces the stickleback's ability to pay attention to other visual stimuli, for example, an approaching predator.

We have developed a model representing the situation of a stickleback confronted with a swarm of water fleas. At any time t its costs in terms of fitness units, result from its hunger state $s(t)$ and confusion while feeding at a capture rate $c(t)$ in a density $d(t)$. The dependence of fitness on hunger state is assumed to be $\alpha s^2(t)$, due to an increased risk of starvation², the costs resulting from confusion are assumed to be $\beta d(t)c^2(t)$, due to a higher vulnerability in the face of predators (greek symbols are used as constants of proportionality to enable adjustment of considered influences). These costs will rise with increasing feeding rate and/or density (our unpublished results). During a given time interval $(0, T)$ the stickleback should minimise its total costs². In other words it will minimise the integral value

$$\int_0^T \alpha s^2(t) + \beta d(t)c^2(t) dt \quad (1)$$

Because the decrease in hunger is proportional to the capture rate we have

$$\frac{ds}{dt} = -\gamma c(t) \quad (2)$$

Capture rate $c(t)$ and density $d(t)$ cannot vary independently, because a high capture rate is impossible at a low prey density. On the other hand $c(t)$ is limited by the minimum time taken to swallow an item of prey; that is

$$(a) \delta c(t) \leq d(t) \quad \text{and} \quad (b) 0 \leq c(t) \leq c_{\max} \quad (3)$$

Therefore the stickleback is able to vary $d(t)$ and $c(t)$ freely in an area under a linear curve reaching a plateau. The predictions deduced from the model will remain true if a dependency similar to Holling's disk equation is assumed. In order to choose the combination of $d(t)$ and $c(t)$ with lowest costs, equality will hold in equation (3a). According to Pontryagin's maximum principle, (1) can be minimised by maximising the hamiltonian¹⁰

$$H(c, t) = -[\alpha s^2(t) + \beta \delta c^3(t) + \gamma c(t)p(t)] \quad (4)$$

with unknown adjoint variable $p(t)$. This gives

$$c(t) = \begin{cases} c_{\max} & p(t) < -\frac{3\beta\delta c_{\max}^2}{\gamma} \\ 2\sqrt{\frac{\gamma p(t)}{3\beta\delta}}, & \text{otherwise;} \\ 0 & p(t) > 0 \end{cases} \quad (5)$$

$d(t) = \delta c(t)$ always.

By solving the system of first order differential equations:

$$\frac{ds}{dt} = -\gamma c(t), \quad \frac{dp}{dt} = 2\alpha s(t) \quad (6)$$

the optimal courses of $s(t)$, and therefore $c(t)$ and $d(t)$, can be calculated. This model leads to the following predictions. First, the stickleback has to start in a high density with a high feeding rate; with decreasing hunger, a lower feeding rate should be achieved with less confusion at a lower prey density (our unpublished results and Fig. 1). Second, if the stickleback's costs of confusion are raised because of a predator presence, a lower capture rate within a lower density will be optimal at the start (Fig. 1). Higher capture rates for some time provide a lower

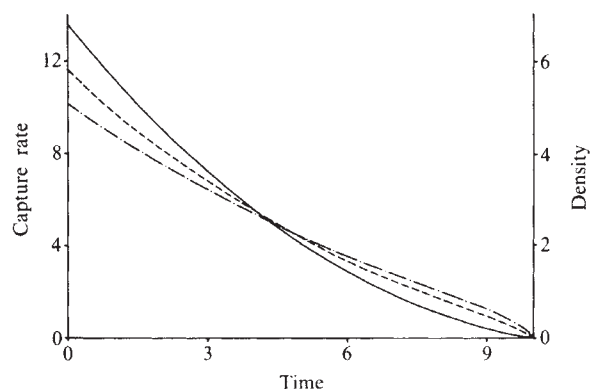


Fig. 1 Optimal courses of capture rates and densities in time (arbitrary units) for different risks of predation. For clarity the same set of curves indicates both the appropriate capture rates, when read with the left scale, and simultaneously the densities, when read with the scale on the right. The more elevated the risk of predation the lower should be the stickleback's initial capture rate and its aimed density. —, low risk; — — —, medium risk; — · —, high risk.

hunger state after this time. Thus sticklebacks initially feeding faster, will have lower capture rates in lower densities afterwards.

Thus in the presence of a predator a hungry stickleback which would normally attack the most dense region of a swarm with a high feeding rate, has to divide its attention between prey and predator and would be expected especially at the start to slow its feeding rate and to attack a region inducing less confusion, that is, a swarm region of a lower density. These qualitative predictions have been tested by the following experiments.

A tank (25.5 × 12.5 × 17 cm) was divided into halves by a partition which included a sliding door. In front of the back wall of one half of the tank there was a transparent plexiglass cell containing the prey, 9 cm from the sliding door. This cell was divided into seven adjacent compartments, each with a base of 1 × 1 cm. These contained 0, 2, 0, 40, 20, 2, and 0 water fleas of approximately equal size (water level 1.5 cm). To simulate the presence of a stickleback¹¹ predator a black silhouette of a European kingfisher (*Alcedo atthis*, 16 cm beak to tail length) suspended on two nylon threads was made to glide over the other half of the tank (start compartment) at a mean distance of 41 cm and at an angle of 50° subtended horizontally. To ensure that the bird stood out from the 'sky' there was a large white canvas sheet 2 m above the tank illuminated from above by a 500 W bulb. Two alternating experiments (with and without predator) were conducted.

Before each trial three water fleas and then a stickleback which had last fed on the previous day were transferred to the start compartment. In the first experiment after the *Daphnia* had been eaten the silhouette was made to glide over the tank and then the sliding door was opened. In the second experiment the bird was not shown. The initial meal of three *Daphnia* ensured that the stickleback reacted to the cell. All fish passed through the door within five minutes and began biting at the prey-containing cell. Attacks on the prey cell were recorded until there was no bite for at least one min. Each fish was used only once. Mean fish length did not differ significantly between experiments.

The sticklebacks in the presence of the predator (disturbed) hesitated longer between door and first bite (median 10.2 s; quart. 7.1 and 18.8) than those in its absence (undisturbed) (median 6.7 s; quart. 5.2 and 7.7; $P < 0.01$, Mann-Whitney U -test, 2-tailed). The undisturbed fish initially attacked the most dense region whereas the disturbed sticklebacks preferentially the least dense region, that is the stragglers (Fig. 2). In subsequent attacks the disturbed fish continued to prefer the two least dense regions and the stragglers in

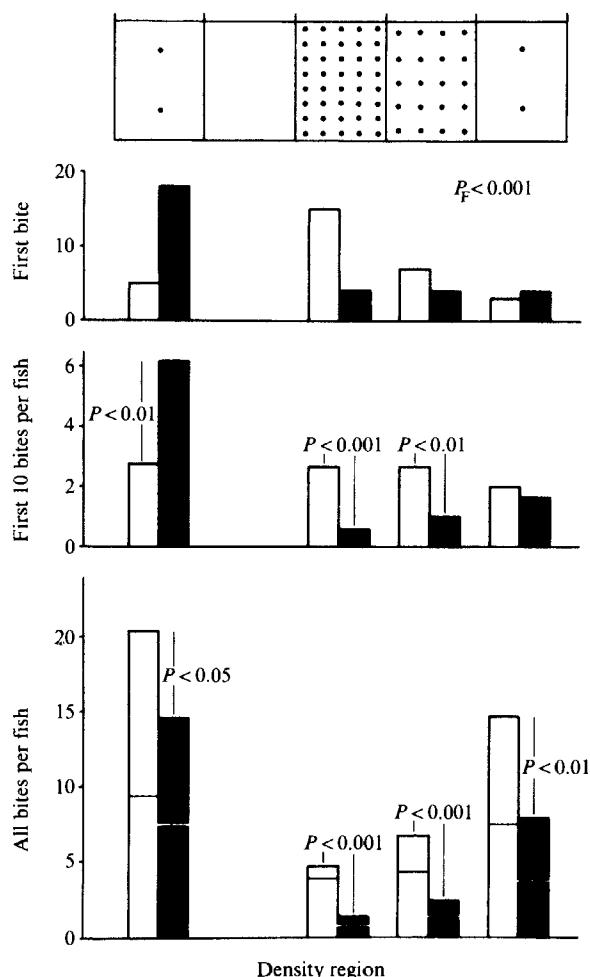


Fig. 2 Attacks in relation to swarm density in the presence (black columns) and absence of the predator model (white columns). Parts of columns below bar represent the first half of total number of attacks. $n = 60$; P after Mann-Whitney U -test, 2-tailed; P_F after Freeman-Halton test.

particular. With decreasing attack readiness the undisturbed sticklebacks changed their initial preference for dense regions to a preference for the least dense regions (Fig. 2). During the first ten bites, each region was attacked nearly equally often, during the first half of the total reaction, there was a slight preference for the less dense regions, while the stragglers were strongly preferred in the second half. Overall, the disturbed fish bit less often (26.0 ± 16.8) than the undisturbed ones (49.7 ± 23.1 , $P < 0.0001$, Mann-Whitney U -test, 2-tailed).

The presence of any predator, for example the kingfisher, should distract the fish's attention when detected. Indeed sticklebacks showed a marked fright response to a dark object about the size of a bird moving above them^{12,13}. It seems improbable that the change in the sticklebacks' preference is an epiphenomenon of a more general adaptation to shock, because electric shock increases the sticklebacks' feeding intensity¹⁴, whereas our disturbed fish decrease their feeding intensity. Also in starlings, surveillance diminished their feeding activity¹⁵. There is good reason to believe that a decrease in motivation to bite against the prey cell acts in a similar way on chosen densities and capture rates as a decrease in hunger (our unpublished results). Thus, as proposed by the model, the changed foraging behaviour is an optimal strategy supplying both needs, namely to pay attention to a predator and with the remaining attention to maximise energy intake by attacking a less confusing swarm density.

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Effect of reduced litter size on the suckling behaviour of developing rats

DURING the past 20 years considerable attention has been drawn to the effects of altered litter size on the growth and development of various mammals. Studies with rodents have shown that increasing litter size permanently reduces body growth by attenuating the rate of cell division and the final number of cells per organ¹⁻⁵. Conversely, reduction of litter size enhances growth and development^{1,6-9}. Altering litter size has also been shown to effect adult behaviour; by elevating emotional reactivity⁹⁻¹², and reducing learning ability¹³ in pups raised in large litters, and by reducing emotionality in pups raised in small litters¹⁰⁻¹². It is not known, however, how quickly behavioural differences may result from alterations in litter size. Moreover, it is unclear whether such behavioural effects are a consequence of nutritional changes or of alterations in the pups' developmental experiences. For example, behavioural differences may be due to alterations in maternal behaviour, as rats nursing small litters spend more time in the nest with these pups, and more time handling and manipulating them^{14,15}. Additionally, varying litter size probably alters within litter competition for the nipples, and this may effect behavioural development as well. Utilising recently developed techniques for the measurement of suckling¹⁶⁻¹⁸ and activity¹⁹ we attempted to discover whether altering litter size directly affects the behaviour of pre-weanling rats. We now report that reducing litter size has the surprising effect of reducing both the willingness of pups to suckle and the vigour of their search behaviour on the mother. Moreover, these effects seem more directly linked to alterations in the litter experience than to nutritional differences.

Sprague-Dawley rats were mated and bred in our own colony. Each experimental comparison was made between two litters of pups born within 3 h of each other. In half the replications the pups were randomised between the mothers and culled to produce a normal size litter ($\bar{x} = 12$, range 8-16) and a small litter of three pups. In the other replications the same litter sizes were used but each pup remained with its natural mother. Culling took place on the day of birth, which was considered day

0. Altogether 36 pups raised in litters of three, and 144 pups raised in 12 natural size litters were studied. Half of the litters were tested on day 5, and the remainder on day 10.

Five hours before the start of testing half the pups from the large litter and randomly either 1 or 2 pups from the small litter were removed and placed in an incubator. These pups constituted the deprived groups. Following deprivation each mother was anaesthetised (Chloropent, 2 ml per kg body weight, intraperitoneally, Fort Dodge Laboratories) and laid supine in a clear plexiglas trough. Both deprived and nondeprived pups were placed individually next to the mother and tested for nipple attachment^{17,18}. Numbers of pups attaching and latency scores were recorded. Each pup was tested for 5 min. Pups were tested both on their own mother and the paired complementary mother.

In addition to this suckling test, a second procedure was used¹⁶. In this test each pup was independently held and gently positioned so that its snout came into direct contact with a presuckled nipple. Attachment latencies were recorded, and a 1 min ceiling was used. Previous research has shown that these are both sensitive tests for the appetitive components of suckling, though the former test (pups on mother) requires that the pups locate the nipples while the latter (pups on nipple) does not¹⁸.

Maternally-directed activity was measured using a digital readout vibrational activity monitor¹⁹ modified to record activity only when the pup was in the immediate vicinity (3 cm) of the mother. Each pup was placed on the sensitive surface with its snout in direct contact with its anaesthetised mother. Activity was recorded for 15 s, which was sufficient time for an accurate measurement of maternally-directed exploration, but was too brief to permit the pups to attach to a nipple.

By 10 days of age serious deficits in suckling behaviour were noted in pups raised in small litters (see Fig. 1). Per cent attachment fell from 100% to 50% (deprived) and from 92% to 0% (nondeprived) on the pups on mother test ($z = 4.49$, $P < 0.001$; $z = 5.42$, $P < 0.001$. Test for the significance of the difference between two proportions²⁰). Testing the pups from the small litters on the mother raising the larger litters had absolutely no restorative effects on suckling. This indicates that the deficit is primary to the pups and is not simply due to some inadequacy of their mother as a target stimulus. Furthermore, the deficits were fully apparent when the pups were placed in contact with highly salient nipples; falling from 100% to 30% (deprived $n = 10$), and from 94% to 25% (nondeprived $n = 8$) respectively ($z = 5.43$, $P < 0.001$; $z = 4.61$, $P < 0.001$).

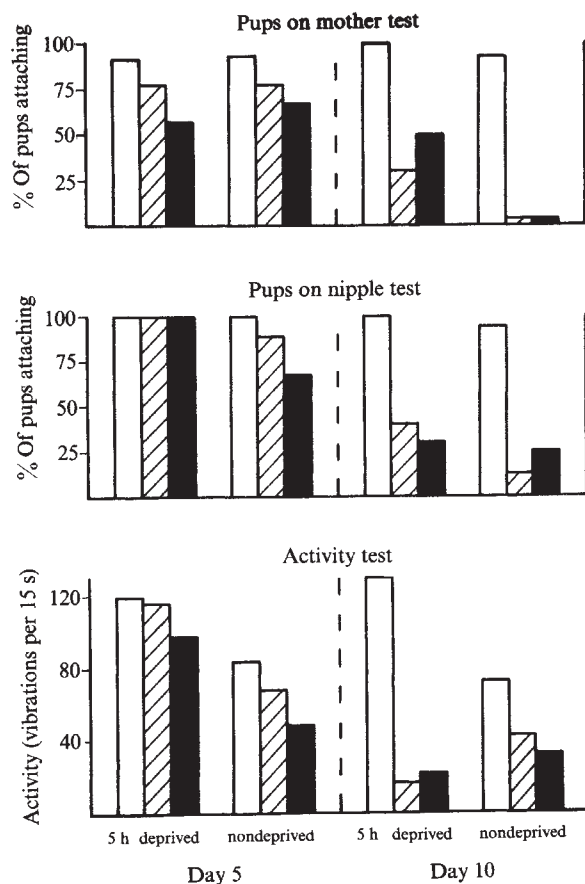
These deficits in nipple attachment were in part mirrored in the activity scores. Normally pups of these ages show an immediate response to anaesthetised mothers, and begin searching the mother's ventrum for nipples. Raising pups in small litters attenuated this behaviour ($F(1,40) = 11.03$, $P < 0.005$; main effect of litter size), though the effect was age dependent, showing up only at day 10 ($F(1,40) = 6.56$, $P < 0.025$; interaction of age $\times$ litter size, day 5: $t(22) = 0.39$, not significant, day 10: $t(22) = 5.80$, $P < 0.001$), and more specifically within the normally very vigorous deprived group ($F(1,40) = 4.24$, $P < 0.05$ interaction of age $\times$ litter size $\times$ deprivation; day 10 deprived: $t(10) = 13.73$, $P < 0.001$, day 10 nondeprived: $t(10) = 2.11$, $P < 0.10$). This observation highlights the fact that the failure of deprived 10-day-old pups to locate and suckle in these test protocols was not just due to an inability to find the nipple (as happens to normal pups on washed mothers²¹) but stems from a general failure to initiate any sort of active search process.

The suckling behaviour of 5-day-old pups however, was less markedly affected by alterations in litter size. Both deprived and nondeprived pups raised in litters of three attached in fewer numbers than normally reared pups when they were required to forage for the nipples of the mothers with which they were reared (deprived: $z = 2.69$, $P < 0.01$; nondeprived: $z = 2.28$, $P < 0.025$). Nondeprived pups also attached in fewer numbers when held in contact with these nipples ($z = 3.58$, $P < 0.001$), although deprived pups attached as readily as controls. Overall,

these deficits in suckling behaviour were less pronounced than those seen on day 10. Moreover, these early deficits were partially ameliorated by testing the pups raised in small litters on mothers raising larger litters, and in most instances significant litter size effects were no longer seen (pups on mother: deprived, $z = 1.21$, not significant; nondeprived, $z = 1.49$, not significant; pups on nipple: nondeprived, $z = 2.07$, $P < 0.05$). In short, the suckling behaviour of 5-day-old pups raised in litters of three is slightly abnormal and exacerbated by the apparently poor mammary development of mothers raising these small litters.

We have demonstrated that raising pups in litters of three markedly affects their pre-weaning suckling behaviour. This finding is of particular importance because we are able to detect the litter size effect at such an early age. However, it raises the interesting question of what factors, nutritive or experiential, create these differences in behaviour. Are the deficiencies in nipple attachment of 10-day-old pups raised in small litters due directly to their elevated body weights (29.5% greater than controls) or to certain experiential differences such as reduced competition in the smaller litter? Fortunately, the general facilitatory effect of reduced litter size on body weight and growth was not absolute, but was relative to the body weight of the mother. Thus pups raised in a litter of three with a small mother frequently weighed no more than siblings raised in a litter of 12 with a much heavier mother. This allowed us to separate experiential effects from nutritive effects. In doing so it became clear that litter size *per se* and not body weight was the crucial factor. Pups reared in litters of three with light mothers (who obtain only normal weight levels) clearly manifest the same deficits in nipple attachment as much heavier pups reared in

Fig. 1 Effects of reduced litter size on suckling (pups on mother and pups on nipple test) and maternally-directed activity. Litters were culled and pups cross-fostered on the day of birth to produce both natural ($\bar{x} = 12$ pups) and small (3-pup) litters. Pups were tested at either 5 or 10 days of age. Open columns, litters of 12 tested on mother raising litters of 12; cross-hatched columns, litters of 3 tested on mothers raising litters of 12; solid columns, litters of 3 tested on mothers raising litters of 3.



litters of three with large mothers. The correlations between litter size and attachment latency for both test protocols were very high even when body weight was excluded from the analysis^{22,23} ($r = -0.935$ and -0.732 pups on mother and pups on nipple test respectively, all P values <0.001). On the other hand, the correlations between body weight and attachment latencies were insignificant when litter size was excluded ($r = -0.234$ and 0.114 respectively, all P values not significant). This result strongly suggests that the litter size manipulation was not affecting suckling simply through its modulation of food availability and body weight. Rather, some experiential aspect of the situation seems to be influencing the pups behaviour. One possibility is that the reduced level of competition in the small litters was not adequate to maintain performance on these suckling tasks, while an alternative possibility is that pups from small litters are accustomed to, and require, a great degree of maternal stimulation before suckling and fail to suckle anaesthetised mothers who do not provide this stimulation.

Overall, it is apparent that raising pups in litters of three attenuates both the appetitive component of suckling, and the vigour with which these pups search their anaesthetised mothers. These differences were small at day 5 and to a large extent reflected differences between the mothers, more than differences between the pups. By day 10 however, deficits in suckling behaviour were marked, and were primary to the pups. Finally, although rats raised in small litters usually weigh more than siblings raised in larger litters, this increase in body weight was not a necessary condition for the disruption in suckling behaviour. It may seem paradoxical for 10-day-old pups from small litters to weigh more than siblings raised in large litters given their apparent hypomotivational state. However, it must be pointed out that the mother's lactational capacity is clearly the rate limiting factor for weight gain in pre-visual pups raised in large litters^{24,25}. Thus, although these pups may be more vigorous in their suckling behaviour, their body weights are in fact limited by restricted food availability.

This study demonstrates that early experience may have significant effects on the ontogeny of ingestive behaviour. What precisely these experiences are, and whether their effects extend beyond the suckling period, remains to be determined. However, this study clearly indicates that 'nutritional' studies relying on litter size manipulations may be producing effects which are not in fact due primarily to changes in nutrition.

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Thermal acclimation in temperate lizards

THERMAL acclimation has been studied most intensively in aquatic ectotherms¹ in which body temperature closely approximates water temperature, which changes slowly with season. As aquatic ectotherms must therefore be active over a range of slowly changing body temperatures, acclimation usually functions as a homeostatic mechanism; following a temperature change in the environment, the organism's metabolic rate is adjusted so that it is returned to near its original level². Some terrestrial animals, particularly reptiles which thermoregulate behaviourally, are subject to considerable daily fluctuations in body temperature³. This difference in the thermal ecology of aquatic ectotherms and terrestrial reptiles, which may cause a difference in the acclimatory responses of the two groups, has not been considered in the work on thermal acclimation in reptiles⁴⁻⁶. Consequently, this work is of little use in predicting the acclimatory responses of reptiles to seasonal temperature changes in the field. We present here evidence that lizards allowed to thermoregulate behaviourally acclimate only at low body temperatures and that the common pattern of acclimation differs from that of aquatic organisms in being determined by requirements of low temperature energy conservation rather than metabolic homeostasis.

Males of four lizard species were used, two from a warm temperature climate (*Podarcis hispanica* and *Psammotromus hispanicus*) and two from a cool temperate climate (*Lacerta vivipara* and *Anguis fragilis*). The experimental design was similar for all species. Lizards were housed in vivaria in a constant temperature room with the temperature adjusted to the mean night time temperature of the season under study. Heat lamps in the vivaria were turned on daily for a period corresponding to the mean photoperiod of the season (Table 1). When allowed access to these lamps, the lizards maintained a relatively constant body temperature which was considerably higher than, and independent of, shade air temperature. At night, with the heat lamps off, the lizards' temperatures fell quickly to ambient. Thus, under these experimental conditions, the lizards were subjected to a fluctuating daily temperature

Table 1 Photoperiod and night time temperature regimes used in the seasonal acclimation study

	Climatic type			
	Warm temperate		Cool temperate*	
	Night time temperature (°C)	Photoperiod (light:dark) (h)	Night time temperature (°C)	Photoperiod (light:dark) (h)
Spring	10	13:11	5	13.75:10.25
Summer	20	14:10	15	15.50:8.50
Autumn	15	11:13	10	10.75:13.25
Winter	5	10:14	—	—

* Cool temperate lizards were studied only in spring, summer and autumn because they are in hibernation, and therefore not exposed to a fluctuating temperature regime, in winter.

regime similar to that experienced under natural conditions. All animals were maintained in this way for at least a month before measurements of metabolic rate were made. Metabolic rate was measured as the rate of oxygen consumption of lizards in small constant pressure respirometers immersed in a constant temperature water bath. All measurements were made on quiescent, post-absorptive animals and therefore approximate to standard metabolic rate. Oxygen consumption rates were measured at 5 degree intervals from 5 °C (the lowest temperature experienced by any animal under the experimental regime) to 30 or 35 °C (the upper body temperatures attained when the heat lamps were on⁷).

For each species, seasonal acclimation had little or no effect on rates of oxygen consumption at the higher temperatures, although significant differences between seasonal groups occur at the lower temperatures for all species (Fig. 1). In other words, differences in metabolic rate do not generally occur when oxygen consumption is measured at temperatures experienced by all groups every day; it is only at temperatures experienced by some seasonal groups but not by others (the lower temperatures) that differences in metabolic rate occur. These seasonal differences in low temperature metabolic rate can be interpreted as mechanisms for energy conservation. In *Podarcis hispanica*

and *Psammodromus hispanicus* (Fig. 1a, b) low temperature metabolic rates are lowest in winter and spring. In the region where these lizards originate, it is at these seasons that there are the longest periods of adverse weather when the lizards cannot come out to bask and feed. In *Lacerta vivipara* and *Anguis fragilis* (Fig. 1c, d) low temperature respiration is less in autumn than in spring or summer; this is possibly a mechanism for reducing low temperature energy expenditure in cool temperate animals entering or about to enter hibernation.

As many reptiles utilise solar radiation to maintain high, relatively constant body temperatures^{8,9}, it is not surprising that little or no thermal acclimation occurs at high temperatures because such body temperatures can be attained each day that the sun shines. To the animal, the main thermal difference between the seasons is the temperature to which its body drops at night, and it is at these low temperatures that seasonal differences in metabolic rate (acclimation) occur. The animal is incapable of feeding at these low body temperatures and it is therefore energetically efficient to effect some measure of energy conservation when the animal has to spend long periods at low temperatures. Thus the common pattern of acclimation in temperate reptiles is determined by considerations of low temperature energy conservation. Some other terrestrial ectotherms also thermoregulate behaviourally¹⁰⁻¹² and it is possible that this pattern of acclimation is widespread in terrestrial animals.

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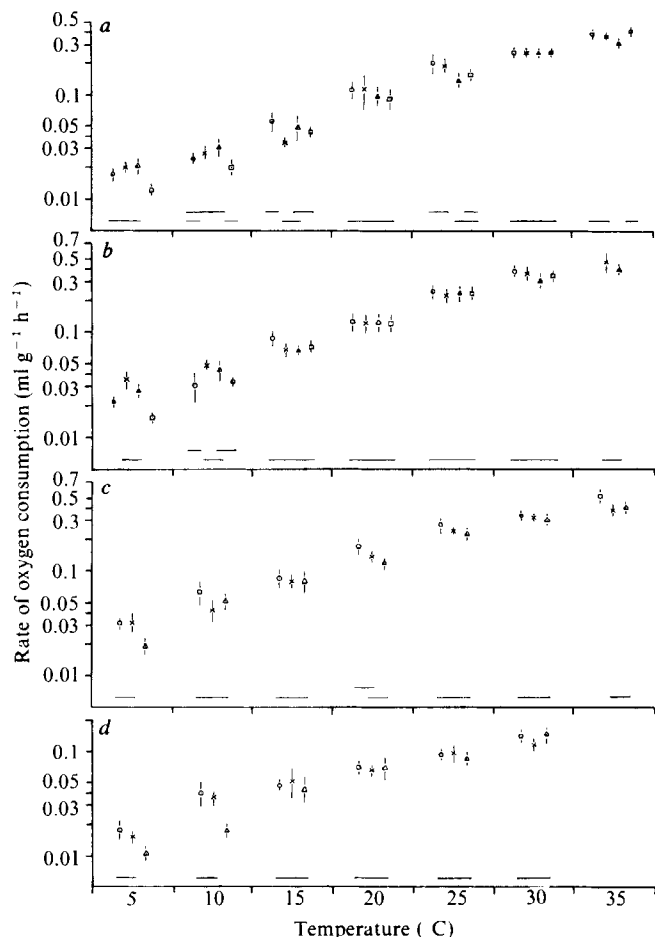


Fig. 1 Rates of oxygen consumption at different temperatures for the different seasonal acclimation groups for males of the four lizard species a, *Podarcis hispanica*; b, *Psammodromus hispanicus*; c, *Lacerta vivipara*; d, *Anguis fragilis*. ○, Mean rates of oxygen consumption for spring acclimation groups; ×, mean rates of oxygen consumption for summer acclimation groups; △, mean rates of oxygen consumption for autumn acclimation groups; □, mean rates of oxygen consumption for winter acclimation groups. The vertical lines above and below each mean are 95% confidence limits of that mean. At each temperature, means not underscored by a common line or a line at the same level are significantly different from each other (analysed by one-factor analysis of variance and Duncan's 5% new multiple range test).

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Feeding, blood glucose and plasma insulin of mice at dusk

MOST omnivorous species exhibit a circadian rhythm of food intake. During one part of the day food is consumed at a rate in excess of immediate metabolic needs, and the surplus is stored to support metabolism for the remainder. Le Magnen and Devos¹, for example, have shown a predominance of lipogenesis in the night-time feeding period of the rat, and pronounced lipolysis during the day, when little food is consumed. A variety of metabolic adaptations exists to match this discontinuous pattern of food intake with relatively constant metabolic use of nutrient², but the question remains whether the hyperphagia at one time of day produces these changes or is a consequence of them. Le Magnen suggests the latter³, at least in the case of increased night-time meal frequency, and in models of feeding, such as that of Toates and Booth⁴, an increased rate of metabolic

clearance of food at night is a considerable factor in promoting increased food intake. On the other hand, eating at a high rate clearly will promote metabolic changes, as bunching of the normal daily intake into a very short period will lead to increased weight gain⁵. A compromise possibility is that a greatly increased rate of feeding at dusk rapidly promotes metabolic changes subsequently favouring a high rate of feeding in the ways suggested by previous authors. The results reported here suggest that such a mechanism may operate in the feeding of mice, in that a great increase in feeding rate around the onset of darkness produces changes in carbohydrate metabolism which may both favour storage of energy and tend to increase subsequent food intake.

All experiments used individually caged female C57 mice, ~100 d old at the start of the experiments, and kept for their entire lives in a temperature-controlled room ($23 \pm 1^\circ\text{C}$), on a constant 12-h light, 12-h dark cycle (lights off 13.00). Unless stated otherwise, standard laboratory food (Spratts rodent breeding diet) and water were available *ad libitum*. Food intake was measured where necessary by regularly weighing feeder inserts in the cages⁶. Blood samples were withdrawn from a tail vein within 2 min of removal from home cage (this technique yields assay results indistinguishable from blood obtained by immediate decapitation). Glucose was assayed in duplicate 100-

Fig. 1 Effects of food restriction on blood glucose (a) and insulin levels (b) at dusk. In the control group (circles), blood glucose falls and blood insulin rises significantly at dusk ($P < 0.01$ in each case, *t*-test with 14 d.f.). There is no significant change in either variable (squares on each graph) if food intake is restricted ($P > 0.1$ in each case, *t*-test with 14 d.f.).

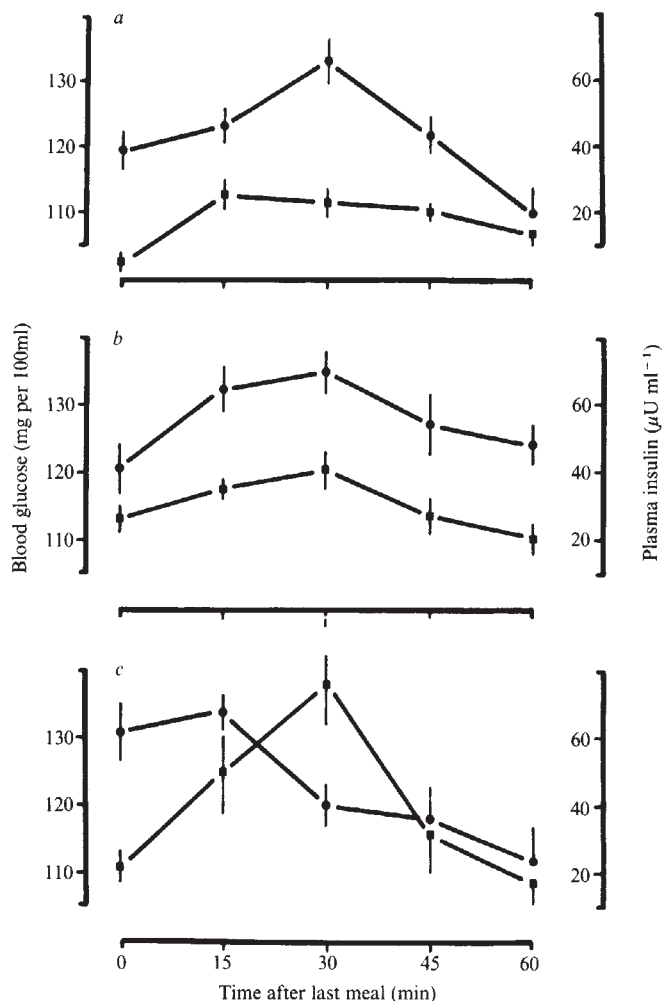
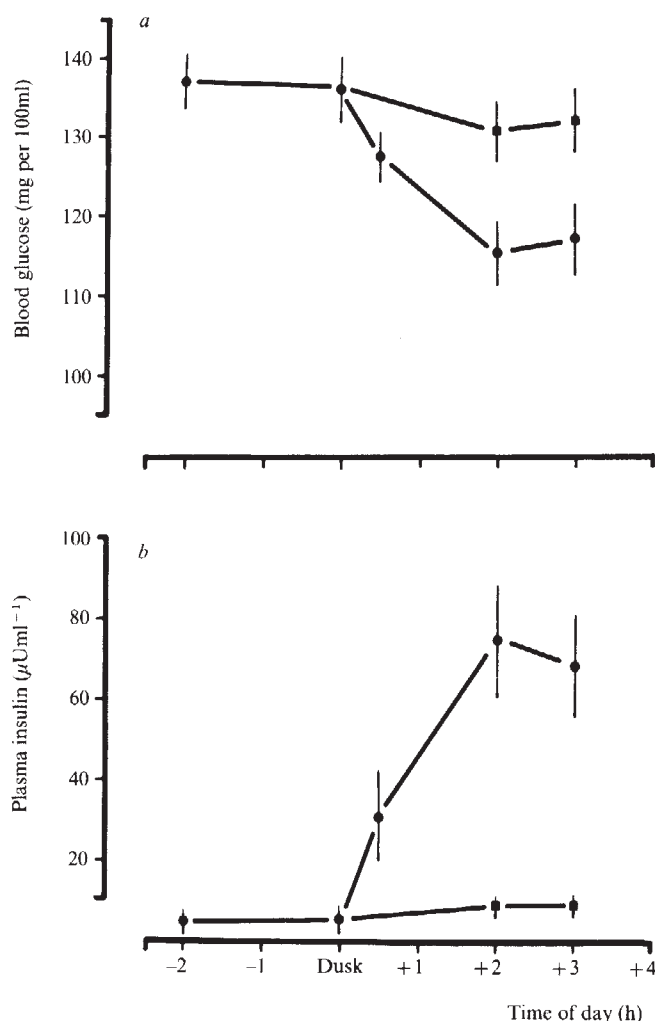


Fig. 2 Effects of combinations of meals on blood glucose and insulin levels. Each graph shows blood glucose (circles) and insulin (squares) levels at 15-min intervals after one (a), two (b) or three (c) meals taken at regular intervals around dusk. Each glucose point is the mean of 12 observations and each insulin the mean of 8. Glucose levels rise significantly only after one meal ($P < 0.01$, *t*-test 22 d.f.), and fall significantly after the third ($P < 0.01$, *t*-test 22 d.f.). Blood insulin levels rise significantly after all combinations of meals, although the magnitude of the rise clearly varies ($P < 0.01$, *t*-test with 14 d.f. in each case).

μl samples of whole blood, using a standard glucose oxidase technique (Glucostat, Worthington). Insulin was measured by radioimmunoassay, again using a standard kit (Radiochemical Amersham), on duplicate 100- μl samples of plasma. A human insulin standard was used, but as all conclusions are drawn from comparisons of insulin levels in different groups of animals, any slight systematic error thereby introduced will not affect their validity. In any one experiment each animal was bled only once, and at least 3 weeks passed between successive bleedings of the same individual, none of which were bled more than three times.

In the first study, the hourly rate of food intake of mice was measured for a few hours before and after dusk, and blood samples were removed at regular intervals for assay. Second, a group of mice were fed normally until 1 h before dusk, and thereafter restricted to their afternoon rate of feeding. Blood glucose and insulin levels sampled at regular intervals were compared with a control group allowed *ad libitum* access to food.

Mice normally eat in meals^{6,7}, and in the final experiment the normal dusk pattern of meal taking was mimicked to examine its metabolic consequence. Mice were allowed *ad libitum* food and water until 2 h before dusk, after which food was removed.

Starting 90 min later, groups of animals were given one, two or three 'meals' by allowing 10-min periods of access to food, separated by 20 min. Animals were observed to eat about the same amount in each period, and the 5% of animals who failed to eat in any or all of the periods were discarded. Blood samples were taken from groups of animals at 15-min intervals after their last meal.

There is a fivefold increase in the hourly rate of feeding at dusk. ($P < 0.001$ t -test with 31 d.f.), and this is closely associated with a fall in blood glucose ($P < 0.01$ t -test with 34 d.f.) and a rise in blood insulin ($P < 0.01$ t -test 14 d.f.). As the rate of feeding subsequently declines, these changes are reversed. If food intake is restricted (Fig. 1), blood glucose levels do not fall, and insulin levels do not rise. In the third experiment, the three 'meals' had quite different consequences. Figure 2 shows that the first meal promotes a rise in blood glucose, with little secretion of insulin, the second a further rise in glucose with a considerable rise in insulin, and the third very high insulin levels, associated with a fall in blood glucose. Insulin levels, however, fall more rapidly after the third meal than after the previous two.

Around dusk, therefore, the rate of insulin secretion in these animals increases to an extent sufficient to depress blood glucose levels in spite of a high rate of feeding. This increase could be due either to some endogenous rhythm of pancreatic activity, or to some direct or indirect effect of the sudden increase in feeding. The disappearance of the changes when food intake is restricted would favour the latter explanation. The high rate of feeding is known⁶ to be due to a very high meal frequency (three per hour), and the final experiment shows that during a series of meals very similar to that taken spontaneously by free-feeding animals, there is a transition between the day-time pattern of elevated glucose with relatively little insulin secretion to the night-time pattern of high insulin secretion, high rate of metabolic clearance of glucose, and presumably storage of energy. The voluntary pattern of feeding adopted by mice at dusk therefore seems to promote metabolic changes leading to more rapid clearance of a meal, which may, as suggested by Le Magnen, and Toates and Booth, facilitate a greater rate of food consumption by shortening the interval between subsequent meals, thus forming a type of metabolic 'positive feedback' of feeding upon itself. There are several, as yet inseparable, ways in which this might occur. Clearly, the activity of eating frequently, the operation of the gut receiving meals in rapid succession, or the metabolic effects of feeding at a high rate provide some additional stimulus to the pancreas, leading to a higher rate of insulin secretion at a given blood glucose level. The pancreatic islets are known to be influenced by both the nervous system and the gut hormones, so that either or both may contribute to the effect⁸.

If such a positive feedback could be shown to operate in other species, such as our own, then its inappropriate activation by frequent voluntary consumption at high rates may facilitate excessive weight gain.

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Cardioactive substances in the monarch butterfly and *Euploea* core reared on leaf-free artificial diet

THE monarch butterfly (*Danaus plexippus*) is one of the best known species which sequester and store cardiac glycosides¹⁻³ obtained directly from their larval food plants. On the other hand *Euploea core*, a related Danaid species feeding on the same family, Asclepiadaceae, is a poor storer of these substances⁴. Cardenolides are absent if the butterflies are reared on plants which do not contain them, and, in the past, all cardioactivity in the insects' body tissues has been attributed to dietary cardenolides. We now report that extracts of both larva and pupa reared on a leaf-free artificial diet⁵ proved cardioactive, slowing the action of an isolated rat heart (Langendorff preparation)⁶ and increasing the amplitude of contraction.

Specimens were extracted by grinding in a mortar with saline. The volume of saline varied depending on the weight of material, and, where possible, volumes were adjusted to give the same proportion of material to saline. The extraction mixture was centrifuged and 0.1-ml aliquots of this supernatant were injected into the perfusion feed line of the isolated rat heart (Langendorff) preparation. The number of hearts used and the weight of material extracted per 0.1 ml of saline are indicated in Table 1. The maximum change in amplitude of contraction is expressed as a percentage of the control value, and the heart rate was measured over 10-30 s after injection and is also expressed as a percentage of control values.

The emetic effect of mascerated specimens and extracts of *D. plexippus* (at all stages of the life cycle) when fed to pigeons is well known^{2,3,7}. The sensitivity of this test is greater than that of the isolated rat heart, as the presence of small amounts of cardiac glycosides in a butterfly extract can produce delayed emesis when administered to the pigeon, although no response may be observed when the same extract is applied to the rat heart⁸.

Extracts of larvae of *D. plexippus* reared on *Asclepias curassavica* produced an effect typical of cardiac glycosides (Table 1 and Fig. 1b), that is, a marked increase in the strength of contraction, seen most clearly in the latter half of the trace. Note that the drop in heart rate, although associated with cardenolide action, is also reminiscent of the effect of potassium ions which may have been present in the extracts, or could equally be due to

Fig. 1 a, Effect of artificial diet-reared *D. plexippus* larval extract on the rate and strength of contraction of the isolated rat heart (each vertical trace represents one heart beat). b, Effect of *A. curassavica*-fed *D. plexippus* larval extract on the isolated rat heart. c, Effect of *A. curassavica* extract on the isolated rat heart. On each figure the horizontal line indicates 10 s (this is omitted in Fig. 2b); extracts were given at the beginning of the 10-s period.

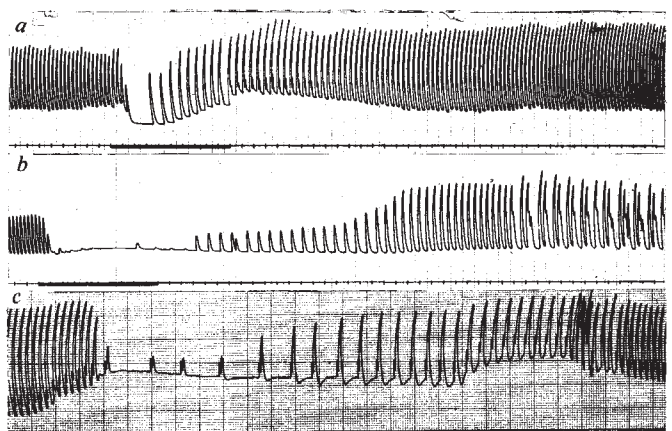


Table 1 Assay of larvae and pupae of *D. plexippus* on the rat heart

		No. of experiments	Average weight of material extracted (mg)	No. of insects extracted	% Change in Rate	% Change in Amplitude
Artificial diet fed	Larvae (2nd instar)	2	283	7	-78	+67
	Larvae (4th instar)	2	249	7	-66	+56
<i>Asclepias</i> fed	Larvae (2nd instar)	1	283	5	-97	+119
	Larvae (4th instar)	1	249	5	-98	+127
<i>Asclepias</i> fed	Pupae	2	161	2	-79	+84

Larvae were starved for 24 h before testing. Diet-reared malformed pupae did not produce emesis when fed to the pigeon whereas pupae reared on *Asclepias* did³.

the insect's own secretions. Figure 1c shows that the extract of the food plant produces a qualitatively similar effect. Comparison of this with Fig. 2c, produced by an extract from a cardenolide-free Asclepiad plant, *Marsdenia erecta*, shows that the latter had no discernible effect on the isolated rat heart. The same negative result was obtained with extracts from the cardenolide-free artificial diet. In contrast, extracts of larvae of both *D. plexippus* (Fig. 1a) and *E. core* (Fig. 2a) reared on the artificial diet, showed cardio-activity. The same effect was produced by extracts of the latter species reared on *Marsdenia* (Fig. 2b). However, the relatively brief slowing of the heart rate and increase in contraction are not typical of the cardenolide response elicited by *Asclepias*-reared material.

For comparison, we tested *Tyria jacobaeae*, an aposematic moth, highly repellent to vertebrate predators^{9,10}, containing large concentrations of pyrrolizidine alkaloids which it sequesters from its food plant¹¹. Neither pupa (Fig. 2d) nor foliage of the food plant on which it was reared (*Senecio jacobaeae*) produced any marked action on the rat heart.

It is unlikely that the cardioactive substance produced by *D. plexippus* is a cardenolide, despite its action on the isolated heart and the fact that some insects are known to secrete them¹², for Roeske *et al.* found no cardiac glycosides in this species reared on plants such as *Gonolobus rostratus* and *A. amplexicanlis*², which lack such substances. They did not, of course, test their

specimens for other cardio-active substances, which we can, nevertheless, assume were present. Furthermore, a substance with a very similar effect, found in *Ipomoea batatas*, and also present in the butterfly *Hypolimnas bolina* fed on it⁴, was demonstrated by Reichstein (in Dixon *et al.*³) not to be a cardenolide.

Most highly coloured insects possess more than one chemical deterrent¹³, frequently a combination of both a primary self-secreted or self-manufactured substance, and a secondary, superimposed, plant-sequestered toxin. Such a primary defence must be particularly important in the case of *D. plexippus*, as the cardenolides which it sequesters and stores, and which so far have been considered by many workers to be its only defence mechanism, are frequently lacking in the foliage of its favourite food plants such as *A. syriaca*¹⁴. Thus, a self-secreted cardio-active substance, even though non-emetic, will make the population much less dependent on the variation and fluctuations characteristic of the secondary plant substances.

Experiments are in progress with various predators and live Danaids reared on an artificial diet. It is essential to use fresh material in these feeding experiments, as, unlike the secondary plant substances, the majority of toxic self-secretions of Lepidoptera are lost or destroyed¹⁵ by storage.

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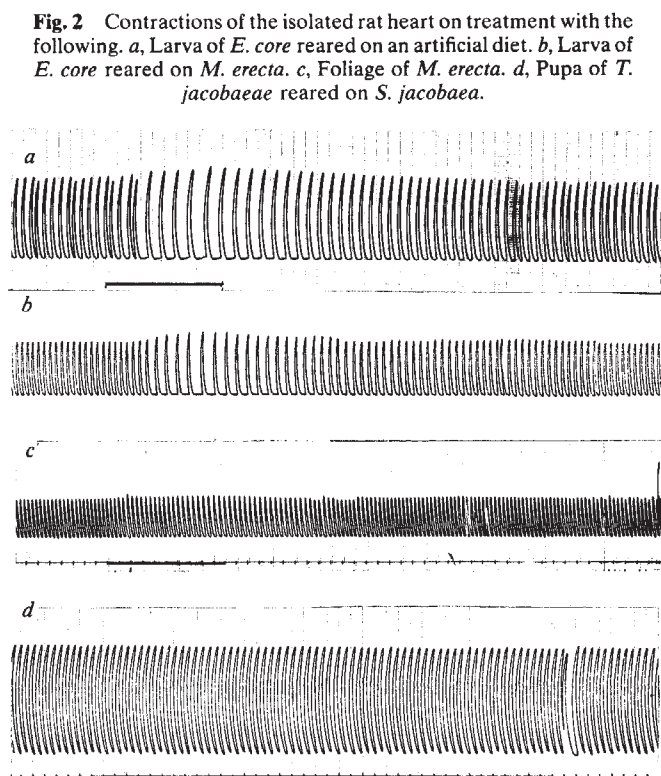
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The relationship between tonicity and flagellar length

THE manner in which a cell regulates the size and number of its organelles is not fully understood. We have studied the regulation of flagellar length. Flagella are organelles involved in cellular motility and sensory transduction¹, and in standardised conditions, the flagella of a given species are constant in length and number. Thus, taxonomists include these characteristics in species descriptions². The organism we are investigating is the unicellular biflagellate green alga *Chlamydomonas reinhardtii*, in which the two flagella are of equal length². If the flagella of *Chlamydomonas* are amputated, they regenerate to their original length in approximately 90 min (ref. 3). If only one flagellum is removed the remaining organelle shrinks while the new flagellum is elongating, until both are of the same length. At this point both organelles proceed to elongate, stopping when the normal length is reached^{3,4}. Resorption of organelles containing microtubules has been shown to occur under a number of conditions in different organisms⁵. Recently it has been shown that the flagella of *Chlamydomonas* can be caused to shorten by a variety of agents such as pyrophosphate⁶⁻⁸, chelators⁶, halothane⁹ and other salt solutions^{6,10,11}. This large variety of substances led us to study the effects of tonicity, *per se*, on flagellar length.

To determine what effect osmotic pressure has on flagellar length, vegetative cells of *Chlamydomonas reinhardtii* were incubated in two salt solutions: sodium chloride and sodium pyrophosphate, and two sugar solutions: mannitol and sucrose, all at a tonicity approximately three times that of normal growth media. Figure 1 shows that the flagella shorten to almost half their original length during the first 60 min, regardless of the solution used to alter the tonicity. Furthermore, the rate of shortening is similar in all cases. Since the only similarity between the various solutions is tonicity (not molarity), we attribute the shortening to the increased osmotic pressure.

After 60 min the flagella of cells which are in salt continue to shorten, until a steady state is reached. However, the flagella of cells which are in sugar begin to regrow, reaching near normal lengths after six h. This regrowth of flagella in the sugar can be prevented by placing the cells in the dark (data not shown). This phenomenon is under investigation.

For any given solution, the length of the flagella, at 60 min, appears to be inversely related to the tonicity of the surrounding medium. This is shown in Fig. 2a and b. Figure 2a presents data for NaCl at various tonicities and Fig. 2b presents data for sucrose at different tonicities.

The experiments in Figs 1 and 2 were of short duration (6 h), and it was of interest to determine if in continuous culture in salt solutions of different tonicities, the cells would adapt to the increased osmotic pressure and possess flagella of normal

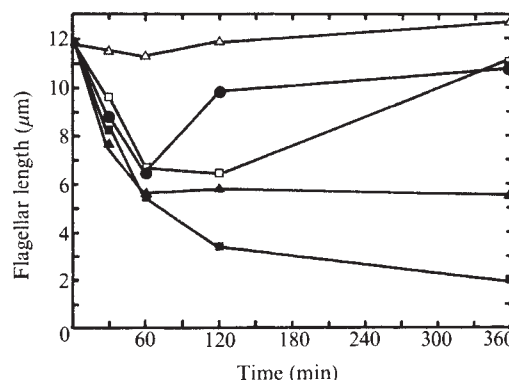


Fig. 1 Change in flagellar length at increased osmotic pressure. Vegetative cells of *Chlamydomonas reinhardtii* were grown on a 12 h light–12 h dark cycle as previously described^{1,12,13}. An equal volume of a cell suspension at a concentration of 3×10^7 cells per ml were mixed with solutions of either mannitol, sucrose, sodium chloride or sodium pyrophosphate (made up in growth media with the pH adjusted to 7.0). At timed intervals thereafter, aliquots of the cellular suspension were removed, fixed with 2.5% glutaraldehyde and flagellar lengths measured as previously described¹³. In addition, aliquots were centrifuged to remove the cells and the osmotic pressure of the supernatant was measured on a model 5100 vapour pressure osmometer (Wescor). □, Mannitol (260 mOsm kg⁻¹; 0.25 M); ●, sucrose (239 mOsm kg⁻¹; 0.2 M); ▲, sodium chloride (240 mOsm kg⁻¹; 0.125 M); ■, sodium pyrophosphate (257 mOsm kg⁻¹; 0.05 M); △, normal growth medium (81 mOsm kg⁻¹).

length. The data presented in Table 1 show that the flagella of cells grown for 2 weeks in various NaCl concentrations were shorter than normal, and the higher the salt concentration, the shorter the flagella. It should be noted that at higher salt concentrations (0.1 M and higher) the rate of cell division was partially inhibited, indicating a stress condition. Thus, flagellar length might be an indicator of osmotic stress on the cells. Furthermore, the short flagella at higher salt concentrations were not a result of mutant selection, since cells washed free of salt regenerated longer flagella (data not shown).

The evidence presented here indicates that the flagella of *Chlamydomonas reinhardtii* respond to changes in osmotic pressure. Higher osmotic pressures (increased by either salt or sugar) causes shortening of the flagella. Thus we conclude that flagellar length is, in part, controlled by the osmotic pressure of the medium. This conclusion is further substantiated by the work of Telser⁹, in which flagella of *Chlamydomonas reinhardtii* were observed to elongate when the cells were placed in distilled water. We have repeated those experiments with similar results. Also, increased osmotic pressure has been shown to lead to the stoppage of the ciliary beat and ultrastructural changes in the cytosol of mussel gill cilia¹⁴. We speculate that some of the flagellar length mutants of *Chlamydomonas reinhardtii*^{15,16} might be osmoregulatory mutants; the cells may be altered in their ability to osmoregulate and therefore have shorter or longer flagella than normal.

We do not yet know the mechanism of shortening due to increased osmotic pressure. One possibility is the action of high tonicities on the microtubules forming the axoneme of the flagella. Protein turnover has been demonstrated in full length flagella, and it appears that the organelle is maintained in a state of dynamic equilibrium¹⁷⁻¹⁹. The loss of water from the cells (due to the osmotic stress) could change the concentrations of solutes in the cell and flagella, which, in turn, would alter the polymerisation and depolymerisation of microtubules.

The regulation of the length of flagella is obviously under the control of complex metabolic processes. Osmotic pressure seems to be an environmental factor which can influence those processes. However, not all flagellar shortenings are due to osmotic effects. Drugs, such as halothane⁹ and herbicides such as amiprofosmethyl⁶ probably cause flagellar shortening by

Table 1 Flagellar length of cells grown in various salt concentrations

Salt concentration (osmolarity)	Flagellar length (μm)	Per cent reduction in flagellar length
Normal medium (81)	13.3	—
0.02 M (90)	6.5	51
0.05 M (116)	4.4	67
0.1 M (196)	1.2	91

Flasks containing 50 ml of culture media, at various salt concentrations, were inoculated with 2×10^6 cells and placed to grow on a 12 h light–12 h dark cycle. After two weeks samples were removed, fixed with glutaraldehyde and flagellar lengths measured as in Fig. 1. Osmolarities were measured as in Fig. 1.

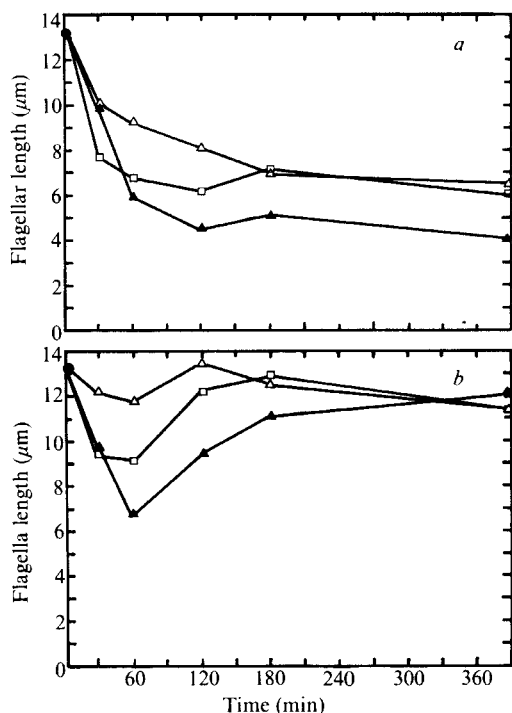


Fig. 2 Change in flagellar length in solutions of different osmolarities. Conditions of cell growth and experimental procedure were the same as in Fig. 1. *a*, Changes in flagellar length in sodium chloride solutions of various osmolarities: Δ, 163 mOsm kg⁻¹ (0.075 M); □, 221 mOsm kg⁻¹ (0.1 M); ▲, 256 mOsm kg⁻¹ (0.125 M). *b* shows changes in flagellar length in sucrose solutions of various osmolarities: Δ, 153 mOsm kg⁻¹ (0.125 M); □, 193 mOsm kg⁻¹ (0.175 M); ▲, 229 mOsm kg⁻¹ (0.2 M). Normal growth medium has an osmolarity of 81 mOsm kg⁻¹. The molarities of the solutions given above are in addition to normal medium.

specific chemical action. Innate cellular properties are also involved in control of flagellar length as illustrated by the alga *Euglena gracilis* which has two flagella of unequal length²⁰.

Our results are significant not only to the cell biologist interested in the physiology of the flagellum, but are also directly related to algal classification, where flagellar length is often included as a species characterisation². Attention should be paid to the salinity of the environment when comparisons are made of flagellar lengths of presumed different species.

After submission of this manuscript a paper which dealt with flagella shortening in *Chlamydomonas* was published²¹. It was reported that 150 mM NaCl caused shortening of flagella while equimolar KCl did not. Our unpublished results show that equimolar KCl will cause flagella to shorten. However, the flagella re-elongate (as in the presence of sugar) and by 90 min (the time at which flagella were measured by Quader *et al.*²¹) the flagella are of normal length. In addition we find that during re-elongation of flagella in the presence of sugar there is a simultaneous accumulation of potassium by the cells. The relationship of potassium to flagellar re-elongation is under study.

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Degenerating nerve products affect innervated muscle fibres

THE main factor controlling mammalian muscle fibre membrane characteristics is the fibre's activity^{1,2}. Other influences may be a trophic factor³⁻⁵, and a loosely termed 'inflammatory effect'^{1,6}. The latter effect is unspecific, appearing when a thread or degenerating nerve is placed over the muscle⁶, after operative procedures near the muscle (ref. 2 and our unpublished observations) and after axonal degeneration within the muscle's own nerve^{1,7,8}. This inflammatory effect may underlie the observations of Cangiano and Lutzemberger⁷ that the innervated fibres in a partially denervated muscle show certain membrane changes similar to those seen on inactive muscle fibres. Such surface changes in inactive muscles have been implicated as the cause of α motoneuronal terminal sprouting⁹, and that they may also occur on innervated fibres in partially denervated muscles provides an explanation for the occurrence of terminal sprouting here too without invoking additional mechanisms. However Cangiano and Lutzemberger's results have been disputed¹⁰. We now report an experiment which demonstrates that degenerating nerve fibres can affect innervated muscle fibres, and that this interaction may well stimulate motoneuronal terminal sprouting.

We produced muscles with afferent nerve fibre degeneration but with normal efferent innervation by cutting the dorsal roots supplying the muscles, peripheral to the dorsal root ganglia. We used the soleus and peroneus tertius muscles of mice weighing 30 g or more. Under chloral hydrate anaesthesia (0.2 ml of 3.5% solution per 10 g body weight) the L4 and L5 spinal foraminae were exposed and as much of the dorsal root ganglia as possible removed, care being taken to avoid damaging the ventral roots. Five to eight days later (when any inflammatory effect should be present¹) the acute experiment was performed, the muscles being examined *in vitro* using methods described previously¹¹. We confirmed the absence of motor denervation by comparing the nerve and directly evoked twitch and 50 Hz, tetanic tensions. We also counted the number of motor units in the muscle using carefully graded stimuli to the nerve and compared this total with that obtained from contralateral control muscles. Muscles with any indication of motoneurone death were discarded (8 muscles from 38 operations). A muscle's acetylcholine sensitivity was taken as the size of its contracture in 5×10^{-4} g ml⁻¹ acetylcholine perchlorate, expressed as a percentage of its maximum directly evoked 50 Hz tetanus. The muscles with their nerves were stained in zinc iodide and osmium tetroxide, and the amounts of degeneration within the nerve, de-afferented muscle spindles, and motoneuronal sprouting were determined.

Controls consisted of the normal contralateral muscles from experimental animals, and also muscles from mice with dorsal roots cut central to the ganglion and hence with intact afferent axons (rhizotomy). This latter group controls for the effects of removing some afferent input to the spinal cord on muscle activity, and also for any effects of nerve degeneration within the spinal cord.

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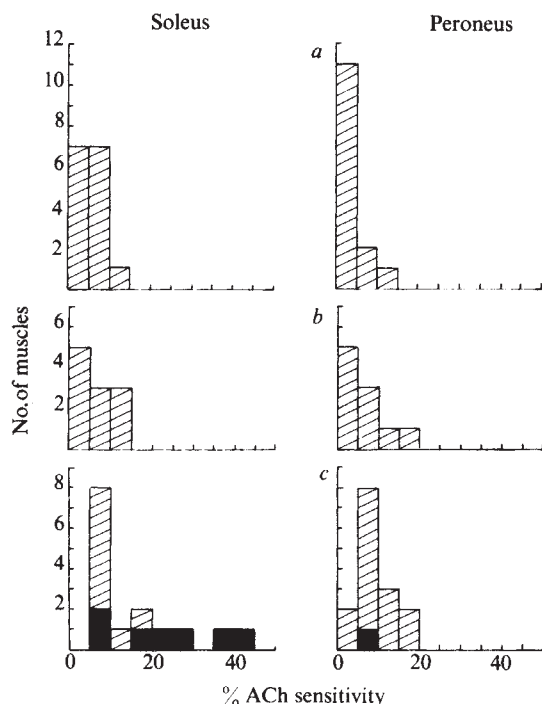


Fig. 1 Histograms of the acetylcholine sensitivities of the various groups of muscles. The rhizotomised solei (b) are not significantly different from the normal control muscles (a). However, the ganglionectomised solei (c) are significantly different from normal muscles ($P < 0.001$ Mann-Whitney U test), and significantly different from the rhizotomised control muscles ($P < 0.025$). The rhizotomised peronei are significantly different from normal controls ($P < 0.05$), as are the ganglionectomised peronei ($P < 0.01$). The two operative procedures do not produce significantly different acetylcholine sensitivities in this muscle, although the mean sensitivity is higher in the ganglionectomies (10 ± 0.5 , $n = 15$ compared with 7 ± 1.6 , $n = 10$). The black portions on the histograms are the results from muscles where more than 40% of the spindles were de-afferented.

Figure 1 shows the acetylcholine sensitivity of the three groups of muscles. The ganglionectomised solei are significantly more sensitive than either of the control groups ($P < 0.025$, Mann-Whitney U test), which are not significantly different from one another. Both the ganglionectomised and rhizotomised peronei are significantly more sensitive than the control normal muscles ($P < 0.01$ and $P < 0.05$, Mann-Whitney U tests). In this muscle the two operations do not produce significantly different effects, though ganglionectomy does produce the higher mean sensitivity (10 ± 0.5 , $n = 15$ compared with 7 ± 1.6 , $n = 10$, mean $\pm$ s.e.m.). Figure 2a shows a scattergram of the relationship between the acetylcholine sensitivity and the amount of de-afferentation within soleus. The correlation ($r_s = 0.525$) is significant ($P < 0.05$). Thus, particularly in the soleus, nerve degeneration products do seem to cause innervated muscle fibres to become acetylcholine sensitive.

Examination of the α -motoneurone terminals in experimental muscles reveals terminal sprouting. Figure 2b is a scattergram of the relationship between terminal sprouting and de-afferentation in soleus, and there is a significant correlation ($r_s = 0.453$, $P < 0.05$). The percentage of terminals with sprouts was 17 ± 5.6 , $n = 16$ in ganglionectomised solei, 6 ± 1.0 , $n = 9$ in rhizotomised solei and 7 ± 2.8 , $n = 10$ in normal solei.

These results support the suggestion that muscle fibre surface changes are a local stimulus to terminal sprouting⁹, and agree with the results of other experiments¹². However, we cannot rule out the possibility that some feature of degenerating nerves might act directly on intact nerve fibres to stimulate terminal sprouting, as seems to be the case for collateral or nodal sprouting¹³. We do in fact see some collateral sprouting in the

intramuscular nerve branches of these de-afferented preparations.

Extensive de-afferentation is required to give significant increases in acetylcholine sensitivity (Fig. 2a) and it is only in such muscles that we see much sprouting (Fig. 2b). However, it is well known that relatively small amounts of partial motor denervation are sufficient to produce large increases in sprouting. In fact, removing only one or two motor units from soleus by selective ventral root damage can produce up to 60% terminal sprouting (data not shown). This might suggest that muscle fibres or motoneurons are especially responsive to motoneurone degeneration. A simpler explanation is that motoneurons arborise profusely within a muscle and pass close to one another's endplates, unlike afferent fibres which do not branch much nor run through the endplate zone. Consequently a single degenerating efferent fibre may produce larger amounts of active substances closer to their sites of action than a single degenerating afferent. A third possible explanation for the apparently greater amount of sprouting after ventral root damage is that denervated muscle fibres might liberate a sprouting agent which could spread through the muscle. This seems unlikely, because in a muscle where transmission is partially blocked, sprouting occurs only from the blocked terminals⁹. If the inactive and hence altered muscle fibres liberated diffusible chemicals which could elicit sprouting then one might expect unblocked terminals to sprout also.

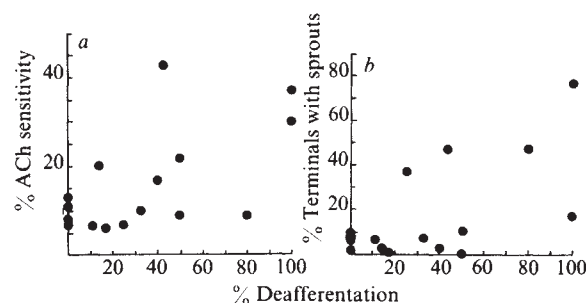


Fig. 2 a, A scattergram of the relationship between acetylcholine sensitivity of solei and the amount of de-afferentation produced in ganglionectomised preparations, r_s (Spearman rank correlation coefficient) = 0.525, which is significant ($P < 0.05$). The degree of de-afferentation was assessed by counting the number of deafferented muscle spindles and expressing this as a percentage of the total number of spindles in that muscle. A normal soleus has 6–8 spindles. Obviously this may only be a rough estimate of the total amount of de-afferentation. This graph demonstrates that large amounts of de-afferentation must be produced to have much effect on the muscle's acetylcholine sensitivity, and this may explain the small size of the differences seen in Fig. 1. Especially in the earlier experiments, in trying to avoid ventral root damage the surgery was over cautious and removed rather little of the ganglia. Histologically, only small amounts of degeneration were seen, and the proportion of de-afferented spindles was small. This problem was particularly acute in the peroneus, because this muscle appears to receive the bulk of its afferents through L5, and this ganglion being harder to remove is usually less damaged. The result was that fewer than half the peronei had any completely de-afferented spindles. Considering only the black squares in Fig. 1 (de-afferentation > 40%), the effect of ganglionectomy is quite obvious. (The rhizotomy operation is relatively easy, and always destroys 100% of the limb's afferent input to the spinal cord.) The particularly small effect of ganglionectomy in peroneus might also be because it contains 'fast' motor units. It has been shown that a fast pattern of muscle activity is particularly effective in inhibiting the development of acetylcholine supersensitivity¹. b, A scattergram of the relationship between terminal sprouting and de-afferentation. There is a significant correlation ($r_s = 0.453$, $P < 0.05$). There is also a significant correlation between the amount of sprouting and the acetylcholine sensitivity of the muscle ($r_s = 0.523$, $P < 0.05$), as is found in certain other circumstances in which sprouting occurs^{9,12}.

Finally, in order to unravel complex innervation patterns (for example, those of the muscle spindle), it is common to use de-afferented or de-efferented preparations. Our experiments have shown that de-afferentation causes efferent sprouting; clearly this may produce misleading descriptions of the normal pattern of innervation in such preparations. Perhaps this explains some of the controversy¹⁴ over the innervation of the muscle spindle.

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Adjuvant-induced thymus-derived suppressor cells of cell-mediated tumour immunity

THE use of immunoadjuvants in enhancing immune responses to various antigens and particularly in tumour therapy is based largely on the concept that an increased level of specific immunity may be achieved by nonspecific stimulation of the immune system¹. However, the tumour-protective effect of adjuvants has not been entirely consistent in all systems studied, and also, an immune suppressive effect of adjuvants has been demonstrated mainly by using *in vitro* assays of cell-mediated immunity². It remains to be determined whether a defect measured by such general tests of lymphocyte function may also represent an indication of depressed cell-mediated immunity against the progressive growth of tumours. Moreover, the target cell in the immune system for adjuvant effect has not been characterised clearly. Our laboratory has made extensive study of immune response to SV40-induced syngeneic transplantable MKSA cells of BALB/c mice^{3–5}. This tumour has been shown to possess tumour-associated antigens which can immunise specifically against the growth of MKSA cells. The role of T lymphocytes in the immune response against SV40-induced tumour-associated antigens was shown by *in vivo* tumour cell neutralisation assay (Winn)⁶ and by *in vitro* ⁵¹Cr release assay⁷. We now report that injection of complete Freund's adjuvant (CFA), one of the most useful adjuvants in enhancing immune responses, markedly inhibited the ability of immune spleen cells to prevent SV40-induced tumour growth in the Winn assay and to lyse SV40-transformed cells in the cytotoxicity assay. Moreover, untreated mice which reject suboptimal numbers of tumour cells die of progressive tumour growth following treatment with CFA and tumour cells; adult thymectomy reverses the suppression caused by CFA; and the target cell for CFA effect is the T lymphocyte. These data suggest that treatment

Table 1 The effect of CFA on growth of SV40-induced tumour and on cell-mediated cytotoxicity against SV40-transformed cells

Treatment	Challenge with MKSA cells* (dead/total)	Neutralisation of MKSA cells† (dead/total)	% Cytotoxicity against C57SV cells‡
None	0/10	0/10	39.2
CFA	9/10	10/10	2.3

This is a representative experiment out of five experiments with similar results.

* CBF₁ mice were inoculated s.c. with 2×10^7 C57SV cells. After 15 d the animals were challenged s.c. with 1×10^5 MKSA cells.

† CBF₁ mice were inoculated s.c. with 2×10^7 C57SV cells. After 15 d the spleens were removed and a T-cell-enriched fraction was prepared as described earlier. The cells were incubated with 1×10^5 MKSA cells, at a 300:1 lymphocyte:tumour cell ratio, for 45 min at 37 °C in a CO₂ incubator and injected s.c. into normal CBF₁ mice.

‡ CBF₁ mice were inoculated s.c. with 2×10^7 C57SV cells. After 8 d a T-cell-enriched fraction was prepared from the spleens of these mice and used in the *in vitro* ⁵¹Cr release assay. The assay was carried out in the wells of flat-bottom microplates. C57SV target cells were trypsinised and 1×10^4 cells and 5 μ Ci Na⁵¹CrO₄ in 0.2 ml RPMI 1640 medium containing 5% fetal bovine serum were added to each well. After 18 h at 37 °C in a CO₂ incubator, the target cells were washed and effector cells were added in a 0.2 ml volume in a ratio of 200:1 lymphocyte:target cell. After 6 h incubation at 37 °C in a CO₂ incubator, the plates were centrifuged at 350g for 5 min and 0.1 ml of the supernatant was removed for counting. Results are calculated from the mean of triplicate samples and expressed as:

% Specific lysis

$$= \left(\frac{\text{c.p.m. } ^{51}\text{Cr released in the presence of immune cells}}{\text{c.p.m. } ^{51}\text{Cr released in the presence of normal cells}} - \frac{\text{c.p.m. } ^{51}\text{Cr released in the presence of 1\% SDS}}{\text{c.p.m. } ^{51}\text{Cr released in the presence of normal cells}} \right) \times 100$$

The ⁵¹Cr released in the presence of normal cells was always either very close or identical to the release in medium and ranged over 5–15%.

with CFA causes depression of specific anti-tumour cell-mediated immunity by activation of suppressor thymus-derived lymphocytes.

Female CBF₁ (BALB/c $\times$ C57BL/6)F₁ mice aged 8–12 weeks (unless otherwise indicated) were used and SV40-transformed cells, C57SV, of C57BL/6 mice. These cells are good targets in the *in vitro* ⁵¹Cr release assay^{7,8} but do not grow *in vivo*. SV40-transformed cells, MKSA, of BALB/c mice grow *in vivo*⁹ but are poor targets *in vitro*. Both cell lines were shown to possess tumour-associated transplantation antigens which can immunise specifically against the growth of MKSA cells^{4,8}.

In the first experiment the mice were inoculated intraperitoneally (i.p.) with 0.5 ml of CFA (0.5 mg ml⁻¹ *Mycobacterium tuberculosis*, Difco) or left untreated, and immediately afterwards they were inoculated subcutaneously (s.c.) with 2×10^7 C57SV cells. (The amount of CFA and the timing of inoculation in relation to C57SV cell immunisation were based on preliminary experiments.) The mice were then divided into three groups. The spleens of the first group of mice were removed 8 d after immunisation^{7,8}, and a T-cell-enriched fraction was prepared using nylon wool columns, as described by Julius *et al.*¹⁰. About 30% of the starting spleen cell population was recovered in the nonadherent fraction; of these, 1–3% (compared to 40–45% in the unfractionated population) were surface immunoglobulin-positive cells, and 0.3% were phagocytic cells (compared to about 5% in the unfractionated population) as judged by latex particle ingestion. The cells were used in the ⁵¹Cr release assay against C57SV target cells. Marked depression of cell-mediated cytotoxic activity was observed in the CFA-treated group as compared to the control group (Table 1). This suppression was demonstrated by using various attacker:target cell ratios and by testing spleen cells at various times after C57SV cell immunisation. The second group of mice was challenged s.c. with 1×10^5 MKSA cells 15 d after immunisation.

Table 2 The effect of CFA-induced suppressor T cells on growth of SV40-induced tumours

Treatment and no. of spleen cells transferred		Challenge with MKSA cells† (dead/total)
Untreated unfractionated	6×10^7	0/10
CFA-treated unfractionated	6×10^7	8/10
CFA-treated unfractionated	3×10^7	1/10
CFA-treated unfractionated	1.5×10^7	1/10
Untreated nylon column	6×10^7	1/10
CFA-treated nylon column	6×10^7	9/10
CFA-treated nylon column	3×10^7	8/10
CFA-treated nylon column	1.5×10^7	1/10
Untreated anti- θ and complement (C)*	6×10^7	2/10
CFA treated anti- θ and C'	6×10^7	0/10
CFA treated anti- θ and C'	3×10^7	1/10
CFA treated anti- θ and C'	1.5×10^7	1/10
CFA treated C' alone	6×10^7	8/10

* The preparation of AKR anti-Thy 1.2 C3H antibody and the treatment of lymphoid cells were carried out according to the technique described in detail in ref. 12. This antiserum lysed more than 97% of C3H thymocytes and 35–40% of C3H spleen cells by trypan blue dye exclusion. The T-cell response of spleen cells to phytohaemagglutinin was abolished after treatment, whereas the B-cell response to lipopolysaccharide was left intact.

† CBF₁ mice were inoculated i.v. with various spleen cell preparation as shown and immediately s.c. with 1×10^3 MKSA cells.

The third group was killed 15 d after immunisation and T-enriched spleen cell populations were mixed with 1×10^5 MKSA cells and injected s.c. into normal CBF₁ mice. All the mice were inspected until death from tumour growth.

In both groups, CFA treatment caused suppression of tumour immunity (Table 1); thus, most CFA-treated mice died of progressive tumour growth after challenge whereas no tumours were observed in untreated animals and no animal died. Similarly, in the Winn assay, inhibition of tumour cell neutralisation activity of lymphocytes from CFA-treated mice resulted in tumour growth and death of all animals as opposed to untreated animals, in which tumour cell neutralisation resulted in complete protection against tumour growth. Similar results were observed at various times after immunisation and by using various lymphocyte:tumour cell ratios. Incomplete Freund's adjuvant did not have such an effect. No animal died if inoculated with CFA alone. These experiments suggested that suppression of specific cell-mediated tumour immunity may be caused by either a defect in T-cell function or by activation of suppressor cells. To test these hypotheses further, the following *in vivo* experiment was carried out. Normal CBF₁ mice were inoculated i.p. with 0.5 ml of CFA or left untreated. Seven days later (a time based on preliminary experiments), the animals were killed and their spleens were either left unfractionated, passed through nylon wool columns to remove B cells and phagocytic cells, or treated with specific anti-Thy 1.2 serum and complement to remove T cells. The various spleen cell populations were inoculated intravenously (i.v.) into normal CBF₁ mice which were then injected s.c. with 1×10^3 MKSA cells (the number of tumour cells which in preliminary experiments was found to result in no tumour growth in normal CBF₁ mice as opposed to a higher number of cells). The results of a representative experiment out of two experiments with similar results are summarised in Table 2. Injection of mice with spleen cells from untreated animals, either unfractionated or enriched for T cells or B cells and macrophages, and challenge with MKSA cells did not result in tumour growth or death of these animals. On the other hand, injection of mice with spleen cells from mice pretreated with CFA, either unfractionated or enriched for T cells, resulted in tumour growth and death of the animals.

Treatment of cells with specific anti-Thy 1.2 serum and complement but not with complement alone abolished the suppressive activity. These results suggested that suppressor T cells were generated in CFA-treated animals and suppressed *in vivo* the immune response against MKSA cells.

A similar experiment was carried out using adult thymectomy, as it was shown that adult thymus may actively regulate the function of T cells¹¹. Normal CBF₁ mice 6–8 weeks old were either sham thymectomised, thymectomised or left untreated. Ten days later the mice were inoculated i.p. with 0.5 ml CFA or were left untreated, and all mice were inoculated s.c. with 1×10^3 MKSA cells. The results of a representative experiment out of three experiments with similar results are summarised in Table 3. Normal mice treated with CFA died of tumour growth as compared to nontreated animals. Similar results were obtained with sham thymectomised mice. On the other hand, thymectomy and treatment with CFA did not result in tumour growth, similar to results with thymectomy without CFA treatment. Thus, adult thymectomy reversed the suppressive effect of CFA, and protected mice from the lethal effect of tumour growth.

Table 3 The effect of adult thymectomy and CFA on growth of SV40-induced tumour

Treatment	CFA	Challenge with MKSA cells* (dead/total)
None	–	1/10
None	+	9/10
Sham thymectomy	–	0/10
Sham thymectomy	+	8/10
Thymectomy	–	2/10
Thymectomy	+	1/10

* CBF₁ mice were treated as shown and described in the text and inoculated s.c. with 1×10^3 MKSA cells.

The present study demonstrates the suppression of anti-tumour cellular immunity by treatment with CFA, as assessed by *in vivo* tumour rejection and *in vitro* cell-mediated cytotoxicity, two immunological functions mediated by T cells. CBF₁ mice challenged with syngeneic SV40-transformed cells and CFA failed to develop effector cells, and instead they developed suppressor T cells which inhibited *in vivo* effector T-cell functions. In contrast, adult thymectomy reversed the suppression caused by CFA, suggesting that the intact adult thymus was necessary for the induction of suppressor cells, that is, the thymus may regulate immune responsiveness by the export of suppressor T cells which regulate other T-cell responses. These studies agree with those demonstrating suppression of *in vitro* immune responses to alloantigens by CFA^{13–15}. In contrast to suppressing effector-cell function to antigens, CFA augmented antibody responses to the same antigens^{16,17}. Those studies suggested that increased helper T-cell activity is favoured by adjuvant. Taken together, these and our own studies suggest that CFA has a differential effect on at least two T-cell subpopulations. Regulation of T-cell function is accomplished by the induction of suppressor T cells that selectively interact with pre-killer T cells while sparing helper T cells. A relevant aspect of tumour immunology suggested by the present study is that there may be quite a delicate balance between the beneficial anti-tumour effects of adjuvants and their immunosuppressive effects. Considerable experimentation seems to be necessary to determine the various factors contributing to this complex situation; therefore, caution should be exercised in using adjuvants for immunotherapy of cancer.

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Characterisation of a tumour-specific antigen on the surface of feline lymphosarcoma cells

In domestic cats, regression of tumours originally induced by inoculation of feline sarcoma virus (FeSV) as well as the resistance to feline leukaemia virus (FeLV)-induced lymphosarcoma in the natural environment are correlated with high titres of antibody to the feline oncornavirus-associated cell membrane antigen (FOCMA)^{1–4}. FOCMA is present on FeLV-producing and nonproducing feline lymphosarcoma (LSA) cells and FeSV-infected fibrosarcoma cells but is absent from FeLV-infected normal cells. This finding is consistent with the interpretation that FOCMA represents an FeLV or FeSV-induced transformation-specific protein^{5–7}. Other workers have initiated studies of the biochemical nature of FOCMA derived from cytoplasmic extracts of cells of a heterologous species (mink) nonproductively transformed by FeSV^{8–10}. However, our primary interest has been to analyse FOCMA expressed on naturally occurring LSA cells from pet cats. We describe here the identification of a hitherto unrecognised protein on the surface of feline LSA cells and cultured transformed feline lymphoblastoid cells which exhibits antigenic determinants in common with FOCMA. The molecular weight of this protein is approximately 70,000. Our data agree with the accumulating evidence that FOCMA is distinct from all FeLV and RD114-coded virion antigens^{4,6–11}.

Cell-surface molecules carrying FOCMA determinants were characterised by methods previously used in studies of murine leukaemia virus-coded cell-surface components of normal and leukaemic murine lymphoid cells: enzymatic radioiodination of viable cells, precipitation with typing serum from detergent lysates, and analysis of proteins in immunoprecipitates by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE)^{12,13}. Correct interpretation of the results obtained was dependent on the availability of monospecific FOCMA typing sera. Therefore, a preliminary comparative analysis of feline sera for antibodies to FOCMA and to FeLV structural protein antigens was made. A panel of selected coded sera were tested for the presence of (1) FOCMA-reactive antibodies by the viable cell membrane immunofluorescence test, (2) FeLV-neutralising antibodies, and

(3) precipitating antibodies to individually purified FeLV-AB virion proteins in radioimmunoassays. We were able to select cat sera (Table 1) which reacted only against FOCMA and not against FeLV structural protein antigens (hereafter referred to as FOCMA typing serum (from a class 6 cat)), sera reacting only against FeLV proteins (class 3 cat), or sera negative for FOCMA or FeLV antibodies (class 5 cat).

Figure 1 shows electropherograms of proteins precipitated from lysates of surface-iodinated primary feline LSA cells by sera from different classes of FeLV-exposed cats. Figure 1a and

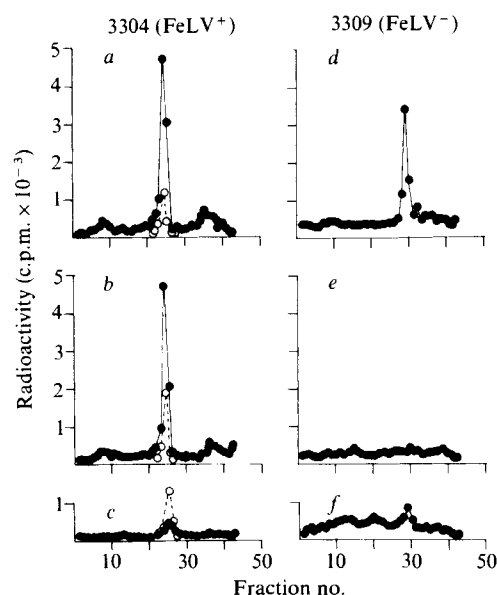


Fig. 1 SDS-polyacrylamide gel (PAG) analysis of ¹²⁵I-labelled surface proteins of primary feline lymphosarcoma (LSA) cells precipitated from cell lysates by antibodies in different classes of feline sera (●). Tumour tissue was obtained from animals at autopsy; thymic LSA cells, FeLV-positive in a fluorescent antibody test for FeLV gs antigens¹⁷, were obtained from cat no. 3304. LSA cells negative for FeLV antigen expression were obtained from a lymph node of a second animal (3309) with multicentric LSA. Preparation of cells and radioiodination of cell-surface proteins were accomplished according to protocols published in detail elsewhere¹². Labelled proteins were solubilised by incubating cells in a cell lysis buffer (CLB) (0.05 M NaCl, 0.02 M Tris-HCl, pH 7.4, 0.5% Nonidet P40, 0.5% sodium deoxycholate) containing 10% Trasylol, a general protease inhibitor (FBA Pharmaceuticals). After dialysis for 16 h at 4 °C, insoluble material was removed by centrifugation. For our double-antibody precipitation procedure, 2 µl of test feline sera was added to aliquots of lysate from 5 × 10⁶ cells (final volume 100 µl in CLB, 2–3 × 10⁶ trichloroacetic acid (TCA)-precipitable ¹²⁵I c.p.m.). The suspensions were incubated at 4 °C for 30 min and the total IgG was precipitated by further incubation (4 °C, 2 h) with an excess of rabbit anti-cat IgG. Precipitates were collected by centrifugation at 3,000g and sequentially washed with 1-ml aliquots of cold (1) 0.5 M NaCl, 0.02 M Tris-HCl, pH 7.4, 0.5% NP40, (2) 1.0 M NaCl, 0.02 M Tris-HCl, pH 7.4, 0.5% NP40, and (3) 0.002 M Tris-HCl, pH 7.4, 0.5% NP40. Labelled polypeptides in immunoprecipitates were analysed by PAG electrophoresis in the presence of SDS and 2-mercaptoethanol in 0.5 × 8.5 cm cylindrical gels (7.5% acrylamide) topped with 0.6 × 1.5 cm stacking gels (4.5% acrylamide) according to the method of Laemmli¹⁸. The ratio of acrylamide to bisacrylamide was 30:0.8. After electrophoresis at 3 mA per gel for 3 h, gels were fractionated and ¹²⁵I in the fractions was determined in a Beckman radioimmuno analyser γ counter. a–c show proteins immunoprecipitated from 3304 LSA cell lysate with FOCMA typing serum 3192A (a), FeLV-neutralising serum 2934C (b) and control cat serum 2263A (c). Proteins immunoprecipitated from 3309 LSA cell lysate with feline sera 3192A, 2934C and 2263A are shown in d, e and f, respectively. ³H-glucosamine-labelled AKR murine leukaemia virus was subjected to co-electrophoresis with immunoprecipitates to serve as a marker for viral gp70 (○).

Table 1 Antibodies in different classes of feline sera

Serum code	Class*	FeLV status†	FeLV neutralising antibody‡	FOCMA antibody§	gp70	RIA titres against ¹²⁵ I-labelled FeLV proteins	
						p30	p15
2934C	3	—	>10	<16	80	320	640
2530A	3	—	>10	<16		1,280	640
2584D	3	—	>10	<16		20	
2768B	3	—	>10	<16			80
2904C	3	—	>10	<16		40	2,560
2263A	5	+	<2	<16	<20	<20	<20
2313A	6	+	<2	32–64	<20	<20	<20
3192A	6	+	<2	128	<20	<20	<20

* Classification of Hardy *et al.*⁴. Class 1 cats are those uninfected cats not exposed to FeLV who do not have neutralising or FOCMA antibody. Classes 2, 3 and 4 cats are uninfected cats exposed to FeLV but not persistently infected with the virus; class 2 cats develop antibodies to FOCMA but no FeLV-neutralising antibodies, class 3 cats develop only neutralising antibodies and class 4 cats develop both types of antibodies. Classes 5 and 6 cats are persistently infected with FeLV; class 5 cats develop no FeLV-neutralising or FOCMA-reactive antibodies, and class 6 cats develop only antibodies to FOCMA.

† Immunofluorescent antibody tests of peripheral blood leukocytes¹⁷.

‡ Titres are the reciprocal of serum dilution giving complete neutralisation of the ability of 40 infectious units of FeLV to induce fluorescent foci in cultured feline fibroblasts.

§ Titres are reciprocals of serum dilutions staining at least 50% of 7.5×10^5 target feline transformed lymphoblastoid cells (FL74)⁶.

|| Titres are reciprocals of dilutions of monospecific rabbit immune sera still precipitating 2.5 ng of corresponding radioiodinated FeLV protein.

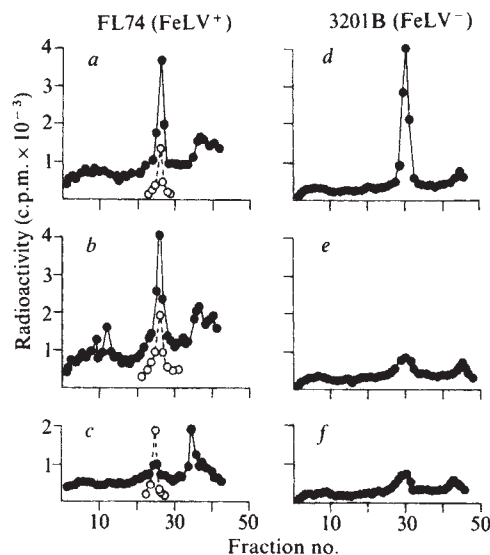
d shows that antibodies in our FOCMA typing serum precipitated a single protein species from LSA cells, regardless of whether these cells showed concomitant expression of FeLV structural antigens. In contrast, immunoprecipitation of a presumed FeLV gp70 molecule with the FeLV-neutralising serum from a class 3 cat was restricted to the FeLV-positive cells (Fig. 1*b, e*). The latter molecule was not precipitated by serum from the antibody-negative class 5 cat (Fig. 1*c, f*). The data shown are representative of results obtained in a series of experiments with biopsied tumour tissue from thymic, multicentric and alimentary forms of feline LSA. Cells from LSAs of the thymus, lymph nodes, kidneys or blood, including tumours of both T- and B-cell origin, were analysed. The molecular weights of the molecules reactive with the gp70 and FOCMA typing sera were estimated to be about 70,000 daltons by virtue of their co-migration with ³H-glucosamine-labelled AKR virus gp70 subjected to co-electrophoresis. A comparison by co-electrophoresis in slab gels of these molecules and several marker proteins of known molecular weight led to a similar conclusion.

These results suggested that the virus-neutralising serum reacted with cell-surface FeLV gp70 and the FOCMA typing serum with a similarly sized molecule possessing different antigenic characteristics. To clarify the reactivities in these sera for FeLV-producing transformed cells a standard target cell line was needed. For this, we chose the well characterised transformed feline lymphoblastoid line FL74 (ref. 5), the standard line used for detection of FOCMA antibodies in cat sera. These cells had been shown to produce FeLV of A, B and C serotypes and to express FeLV envelope and core protein antigens on their surface^{5,14}. A second transformed feline lymphoblastoid cell line (FOCMA-positive but not expressing FeLV antigens) recently established in our laboratories was also studied as a control (W.D.H. Jr *et al.*, in preparation).

In initial experiments (Fig. 2), using a radioimmuno-precipitation assay as described above, we verified that our FOCMA-reactive and FeLV-neutralising sera recognised cell surface molecules on FL74 cells which were similar to those detected on freshly biopsied tumour tissue. That the FOCMA-specific and FeLV-neutralising cat sera were in fact recognising different 70,000-dalton molecules on these cells was established in absorption experiments. Absorption of FOCMA typing serum with normal cat lymph node cells (cells from six animals were tested, including one replicating FeLV), cultured uninfected or FeLV-producing feline lung fibroblasts (FLF), or cultured heterologous mink lung fibroblasts (CCL64)¹⁵, had a negligible effect on precipitation of FOCMA. However, absorption with LSA cells (three samples of FeLV producers

and four FeLV nonproducers) or heterologous mink lung fibroblasts nonproductively transformed by FeSV (64F3CL7 cells)¹⁵ led to a sharp decrease in precipitating activity, such that electropherograms resembled that obtained with antibody-negative cat serum (for example, Fig. 2*c*). Conversely, absorption of precipitating activity in the FeLV-neutralising serum was achieved with FeLV-infected normal feline lymph node cells, cultured FLF cells and LSAs, but not with FeLV-uninfected cells of those types. In other experiments, complete absorption

Fig. 2 SDS-PAGE analysis of ¹²⁵I-labelled surface proteins of cultured transformed feline lymphoblastoid cells precipitated from cell lysates by antibodies in different classes of feline sera. The transformed feline lymphoblastoid cell lines studied were FL74, producing FeLV of subgroups A, B and C, and 3201B, an FeLV nonproducer line established in this laboratory from a naturally acquired feline lymphosarcoma (W.D.H. Jr *et al.*, in preparation). The cells were maintained as suspension cultures in T-75 plastic flasks (Falcon) using McCoy's medium and 15% fetal bovine serum (FBS). Cell surface proteins were radioiodinated, solubilised, immunoprecipitated and electrophoresed as described in the legend to Fig. 1. *a–c* show polypeptides immunoprecipitated from an FL74 cell lysate with FOCMA typing serum 3192A (*a*), FeLV-neutralising serum 2934C (*b*) and control cat serum 2263A (*c*). Polypeptides immunoprecipitated from 3201B cells with feline sera 3192A, 2934C and 2263A are shown in *d, e* and *f*, respectively. ●, ¹²⁵I c.p.m.; ○, ³H-glucosamine-labelled AKR gp70.



of reactivity in the FeLV-neutralising serum could be achieved with a 70,000 dalton fraction from purified FeLV obtained by gel filtration in the presence of 6 M guanidine hydrochloride (GuHCl)¹⁶, whereas fractions containing p30, p15 and p12 had negligible effects. No absorption of reactivity in the FOCMA-specific serum was achieved with any of these fractions of virus in a parallel experiment. The above results indicate that feline LSA cells express on their surface a 70,000-dalton molecule carrying FOCMA which can be distinguished serologically from cell-surface antigens related to FeLV structural proteins.

FOCMA is also induced by FeSV infections, and evidence suggests that in this instance FOCMA may actually be encoded in the FeSV genome⁹. By gel filtration in 6 M GuHCl of proteins from mink cells nonproductively transformed by FeSV, Stephenson *et al.* detected a protein of 85,000 daltons (p85) expressing FeLV p15 and p12 and FOCMA antigens⁹. Pulse-chase experiments showed that this protein is cleaved to give a 65,000-dalton species containing FOCMA and a 25,000-dalton species containing p15 and p12. In addition, Sherr *et al.* described a phosphorylated polypeptide in pseudotype feline sarcoma virus virions (pp85) that seems to be analogous to the p85 precursor described above¹⁰. Surface molecules of FeSV-transformed nonproducer mink cells (64F3CL7) carrying FOCMA were analysed by radioiodination, immunoprecipitation and SDS-PAGE (Fig. 3). In this case, two protein species were precipitated by FOCMA antiserum, and their mobilities in gels corresponded to the intracellular p85 and p65 species described previously. Neither species was precipitated by the control cat serum (Fig. 3a). Identification of the more slowly migrating species as p85 was supported by our finding that it was precipitated with rabbit antiserum to FeLV p12 (Fig. 3b) or p15 (not shown) but was not reactive with anti-FeLV p30 (Fig. 3b). In a control experiment no protein species from similarly

labelled mink cells nonproductively transformed by Kirsten murine sarcoma virus was precipitated by the anti-FOCMA serum (Fig. 3c). These results suggest that the FOCMA-associated p85 and p65 molecules previously described in FeSV-transformed mink cells are associated with the cell membrane. It is likely that the p65 on 64F3CL7 cells is biochemically similar if not identical to the 70,000-MW species described on feline LSA cells. However, in an analysis of many primary feline LSAs we have not detected a larger species (that is, p85) containing both FOCMA and FeLV *gag* gene-coded antigens. This suggests that expression of a FOCMA-associated p85 species may be a unique feature of FeSV-transformed cells, and possibly of 64F3CL7 cells in particular.

The biological function of the FOCMA protein is unknown. Purification and further biochemical analysis of this protein should contribute to a better understanding of initiation and maintenance of neoplastic transformation.

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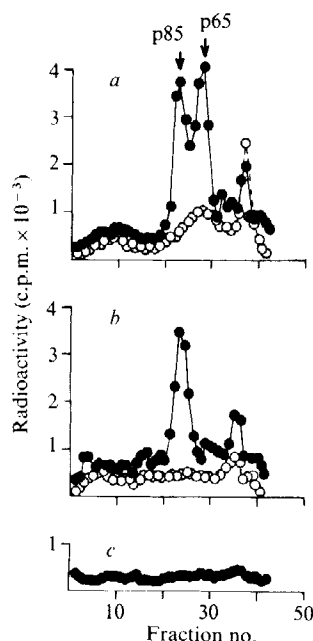
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Fig. 3 SDS-PAGE analysis of ¹²⁵I-labelled surface proteins of nonproducer mink cell clones transformed by FeSV (64F3CL7) or by the Kirsten murine sarcoma virus (64JI) precipitated from cell lysates by antibodies in cat sera and immune rabbit sera. The cells were maintained as monolayers in plastic T-75 flasks (Falcon) using Dulbecco's high glucose medium and 15% FBS. Cell surface proteins were radioiodinated, solubilised, immunoprecipitated and electrophoresed as described in the legend to Fig. 1. *a*, Polypeptides immunoprecipitated from 64F3CL7 cells with FOCMA typing serum 3192A (●) and control feline serum 2263A (○); *b*, polypeptides precipitated from this lysate with rabbit anti-FeLV p12 (●), anti-p30 (○); *c*, proteins precipitated from a lysate of 64JI cells with 3192A serum.



Cytogenetic mapping of the trisomic segment of chromosome 15 in murine T-cell leukaemia

A NONRANDOM cytogenetic change in the form of trisomy of chromosome 15 is associated with mouse T-cell leukaemia induced by various agents, such as Gross, Rad and Moloney leukaemia viruses, X rays and a chemical carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA)¹⁻⁴. The uniformity of the chromosomal change and its apparent independence of the inducing agent suggest that it plays an important and perhaps decisive part in the neoplastic transformation of the mouse T lymphocyte. During our studies of the role of chromosome 15 and related factors in lymphoma development, we have investigated whether any particular region of the chromosome needs to be duplicated. Using DMBA to induce T-cell leukaemias in

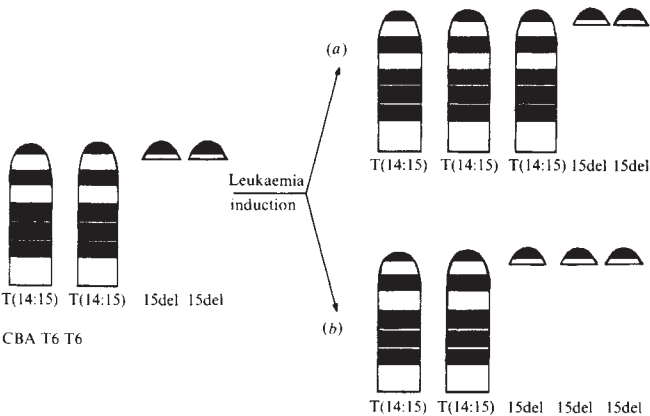


Fig. 1 Schematic illustration of two alternatives (a and b) for chromosome 15 trisomic T-cell leukaemias in CBAT6T6 mice.

mice carrying chromosome 15 translocations, we have found, and now report that the duplicated chromosome segment is located distal to the T6 breakpoint of chromosome 15.

T-cell leukaemias were induced by feeding DMBA to female and male CBAT6T6 mice (Weizmann Institute) as described elsewhere⁵. Overt leukaemias were transplanted subcutaneously to CBA recipients of the opposite sex. The chromosomes of leukaemic cells of the first passage generation were analysed by G banding. Banded metaphase plates were prepared from subcutaneous tumours, spleens and bone marrow by a slight modification of Wang and Fedoroff's procedure⁶. Magnified photographs were used for karyotype analysis, and trisomic chromosomes were also identified under the microscope in metaphase plates with high banding quality. Chromosome identification followed the nomenclature of the Committee of Genetic Standardization⁷. Because all leukaemic cells originated from donor CBAT6T6 mice, they could be distinguished easily from host cells by the double criteria of the sex chromosome and the T6T6 markers. The CBAT6T6 mouse carries the reciprocal translocation T(14;15)6 (ref. 8). Chromosome 15-trisomic T-cell leukaemias were expected to conform to one of the two patterns illustrated schematically in Fig. 1.

Chromosome counts of six tumours are summarised in Fig. 2. Five out of six leukaemias were aneuploid; tumours with a modal number of 41 chromosomes and tumours with a modal number of 42 and 40 + 1 bi-armed (total 42) chromosomes. Only one tumour (mice 2 and 3) had a modal number of 40 chromosomes. Table 1 shows the results of banding analysis. All five aneuploid leukaemias with a modal number of 41 and 42

Fig. 2 Chromosome (Chr.) constitution of cells originating from subcutaneous (s.c.) tumours, spleens and bone marrow of CBA mice inoculated with DMBA-induced CBAT6T6 leukaemic cells.

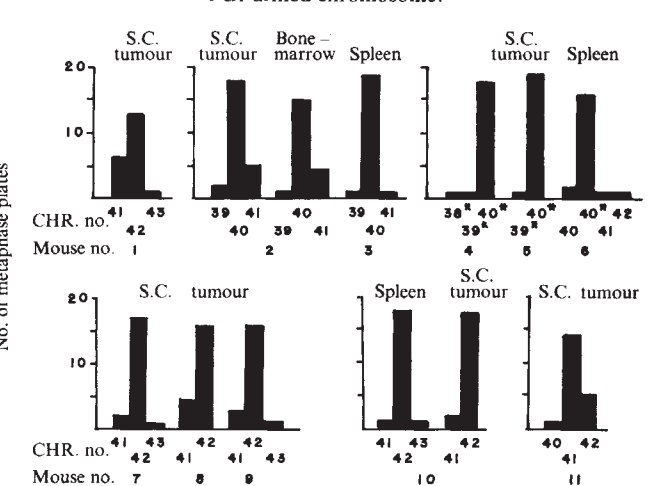


Table 1 G-banding analysis of leukaemic cells with aneuploid chromosome constitution

Mouse no.	Modal chromosome no.	No. of plates analysed by banding	Trisomy of chromosome†
1	42	14 + 8*	T(14;15)6
2	40	20 + 10*	
5	40 + 1B	15 + 15*	T(14;15)6
9	42	10 + 5*	T(14;15)6
10	42	12 + 4*	T(14;15)6
11	41	10 + 8*	T(14;15)6

B, bi-armed. The bi-armed chromosome was formed by centromeric (Robertsonian) fusion between one of chromosomes T(14;15)6 and chromosome 12.

* Karyotyped from magnified photos.

† The second trisomy involved mostly chromosomes 2, 6 and 19.

chromosomes had a trisomy T(14;15)6 (Fig. 3). The second trisomy of tumours with a modal number of 42 chromosomes was distributed randomly, involving mostly chromosomes 2, 6 and 19. The banding pattern of the single tumour with a modal number of 40 chromosomes was indistinguishable from a diploid CBAT6T6 cell.

These results show that the gene(s) that tend to be duplicated during DMBA leukaemogenesis are located distal to the T6 breakpoint of chromosome 15. The consistency of the change further emphasises the specific role of genes associated with

Fig. 3 G-banded karyotype and metaphase plate of a leukaemic cell originating from a female CBAT6T6 mouse and inoculated s.c. into a male CBA mouse. Note the presence of trisomy T(14;15)6 (arrows). The second trisomy involved chromosome 6.



chromosome 15 in T-cell leukaemia. However, the occurrence of a diploid leukaemia in one tumour shows that the change is not exclusive. This agrees with previous findings on AKR and the RadLV leukaemias^{1,2}. In possible analogy with the present work, a specific chromosome aberration affecting one chromosome 14 was found in human B-cell derived neoplasias³, suggesting that certain chromosome segments carry genes that are critical to the neoplastic growth of given target cells.

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An inducible gene involved in commitment of lymphocytes to transform

CONCAVALIN A (Con A) stimulates nondividing lymphocytes (in G_0) to transform and enter the division cycle¹. Within 3 h a significant proportion of the cells become committed to transform and do so after the removal of Con A (ref. 2). When the translation of mRNA into protein is inhibited (by low concentrations of anisomycin) lymphocyte transformation is also blocked³. Anisomycin has proved particularly useful in studying lymphocyte transformation because its effect is reversible: in the presence of Con A it holds the cells in G_0 , apparently without adverse effects, and the cells transform following its removal³. Using anisomycin it has been shown that Con A induces the synthesis of factor(s) which commit lymphocytes to transform³. I have examined the stability and nature of the 'transformation signal', again exploiting the reversible effect of anisomycin, and report here that on binding to the surface of nondividing lymphocytes, Con A induces the transcription of a short-lived mRNA. This mRNA encodes a 'transformation factor' which, when translated, commits lymphocytes to enter the division cycle; extracellular Con A is no longer required.

Spleen cells from BALB/c mice (6–10-week-old males) were cultured in RPMI medium (see legend to Table 1). Transformation was measured by the incorporation of tritiated thymidine (^3H -TdR) into cellular DNA over 48–50 h; the stimulation index was calculated as the ratio of c.p.m. in Con A-treated cultures to c.p.m. in control cultures. The mitogenic response to Con A peaked at $1\ \mu\text{g ml}^{-1}$ and this concentration was used routinely. Anisomycin ($10\ \text{ng ml}^{-1}$) was used to hold Con A-treated cells in G_0 for the first 24 h in culture, after which extracellular Con A was removed using α -methyl-D-

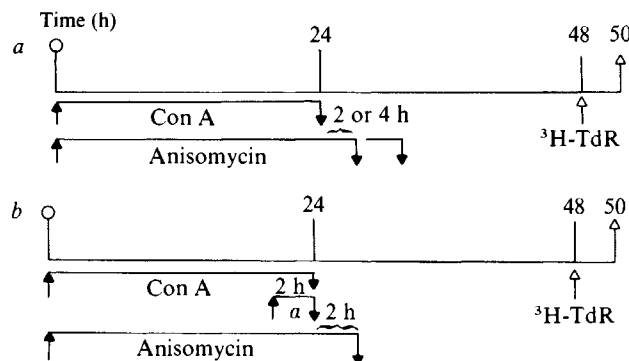


Fig. 1 Plan of experimental procedures described in the text. *a*, The $t_{1/2}$ of the transformation signal was estimated by treating BALB/c spleen cells with Con A ($1\ \mu\text{g ml}^{-1}$) plus anisomycin ($10\ \text{ng ml}^{-1}$) for 24 h. Con A was removed at 24 h using α -methyl-D-mannoside (50 mM), and anisomycin was continued for a further 2 or 4 h before it also was removed. The cells were then cultured in normal medium and ^3H -TdR was added at 48 h for 2 h to assess DNA synthesis. The cells were collected at 50 h. *b*, The possibility that the transformation signal might be mRNA was examined by culturing BALB/c spleen cells with Con A ($1\ \mu\text{g ml}^{-1}$) plus anisomycin ($10\ \text{ng ml}^{-1}$) for 24 h. At 22 h α -amanitin ($10\ \mu\text{g ml}^{-1}$) was added to inhibit mRNA transcription for 2 h and mRNA translation was then inhibited by anisomycin for a further 2 h after the removal of Con A plus α -amanitin. The cells were subsequently cultured and labelled as above. α = α -amanitin.

mannoside³. Additional experimental procedures are summarised in Fig. 1.

The stability of the transformation signal was estimated by continuing anisomycin for various periods after the removal of Con A. The cells recovered and transformed when anisomycin was continued for 2 h after Con A removal but when it was continued for 4 h after Con A removal cell transformation fell by 60% (Table 1). This indicates a half life ($t_{1/2}$) of approximately 3–4 h for the transformation signal (in the presence of anisomycin). This relatively short $t_{1/2}$ suggests that it must be continually expressed in Con A-treated cells held in G_0 for 24 h (by anisomycin) because the cells transform following the simultaneous removal of mitogen plus inhibitor³. Continuous production of the transformation factor presumably depends on the continued presence of Con A, as the ability of the cells to transform fell rapidly 2 h after the removal of Con A (Table 1).

α -Amanitin, a selective inhibitor of mRNA transcription^{4,5}, was used to examine the possibility that the transformation signal might be a species of mRNA transcribed in response to Con A. If the signal is Con A-dependent mRNA, with a $t_{1/2}$ of 3–4 h, it follows that cell transformation should decline markedly when mRNA transcription (in the presence of Con A) and translation (in the absence of Con A) are inhibited sequentially for 2 h each. Again, spleen cells were cultured with Con A plus anisomycin for 24 h. α -Amanitin ($10\ \mu\text{g ml}^{-1}$) was added to the cultures at 22 h and removed at 24 h, together with the Con A (Fig. 1b). Anisomycin was continued for a further 2 h before it also was removed. Experimental controls showed that continued exposure to α -amanitin blocked transformation, whereas cells recovered and transformed when exposed to either α -amanitin at 22–24 h or continued anisomycin for 2 h after Con A removal (Table 2). However, transformation fell by 50% in those cells treated with α -amanitin 2 h before and anisomycin 2 h after the removal of Con A. In other words, the sequential inhibition of mRNA transcription and translation for a total of 4 h resulted in a 50% drop in cell transformation, consistent with the transformation signal being mRNA.

Interpretation of these results clearly depends on the actions of the two antibiotics α -amanitin and anisomycin. In cell-free systems, each has been shown to be highly selective: anisomycin blocks peptidyl transferase activity on 80S ribosomes⁶ and α -amanitin inhibits RNA polymerase II^{4,5} (mRNA synthesis). At very high concentrations ($\geq 200\ \mu\text{g ml}^{-1}$) α -amanitin also

Table 1 Recovery and transformation after prolonged anisomycin inhibition for 1, 2 and 4 h after removal of Con A (see Fig. 1a)

Treatment	³ H-TdR incorporation (c.p.m.)	Stimulation index
Untreated control	2,645	—
I Con A throughout	48,273	18
I Con A + anisomycin throughout	3,267	1.2
II Con A removed at 24 h	44,842	17
II Con A + anisomycin, removed at 24 h	39,287	15
II Con A + anisomycin, removed at 25 h	48,346	18
II Con A + anisomycin, removed at 26 h	42,375	16
II Con A + anisomycin, removed at 28 h	17,290	6.5

BALB/c spleen cells exposed to Con A ($1 \mu\text{g ml}^{-1}$) and anisomycin (1 ng ml^{-1}): the $t_{1/2}$ of the transformation factor was determined by continuing anisomycin inhibition for various periods after removal of Con A at 24 h. Washed spleen cells from a single mouse were diluted to $5 \times 10^6 \text{ ml}^{-1}$ with RPM1 plus 100 IU ml^{-1} penicillin and $100 \mu\text{g ml}^{-1}$ streptomycin. Aliquots ($100 \mu\text{l}$) were dispensed into flat-bottomed wells of a microtitre tray and cultured in a humidified atmosphere of 10% CO_2 in air at 37°C . After removal of Con A (group II) the medium was replaced with Con A-free medium plus α -methyl-D-mannoside (50 mM) to displace any cell-bound Con A (ref. 3). At 25 h the medium was replaced with normal medium. Anisomycin (10 ng ml^{-1}) was added to the replacement media for 1, 2 or 4 h after the removal of Con A; thereafter, it was replaced with normal medium. Transformation was assessed by measuring DNA synthesis using standard techniques after labelling each culture with $2 \mu\text{Ci}$ [$6\text{-}^3\text{H}$]thymidine (specific activity 5 Ci mmol^{-1}) for 2 h at 48–50 h. Each value represents the mean of four cultures.

inhibits RNA polymerase III⁷ (tRNA and 5S RNA synthesis). Intact cells are reputed by some to be impermeable to α -amanitin and this seems to be true for rat hepatoma cells⁸. However, this is not a generalised phenomenon and normal rat liver cells *in vivo*⁸, adrenal cortical cells⁹ and lymphocytes *in vitro* (this work) are all sensitive to α -amanitin at concentrations known to block mRNA synthesis selectively. α -Amanitin may also indirectly affect rRNA synthesis which seems to depend on

Table 2 Recovery and transformation after the sequential inhibition of mRNA transcription (by α -amanitin) and translation (by anisomycin) for 2 h each (see Fig. 1b)

Treatment	³ H-TdR incorporation (c.p.m.)	Stimulation index
Untreated control	550	—
I Con A	52,000	95
I Con A + α -amanitin throughout	480	0.9
I Con A + anisomycin throughout	4,108	8
II Con A	34,708	63
II Con A + α -amanitin for 2 h, at 22–24 h	29,261	53
II Con A + anisomycin continued for 2 h after Con A removal	28,100	51
II Con A + α -amanitin (2 h at 22–24 h) + anisomycin (continued for 2 h after removal of Con A + α -amanitin)	13,307	24

BALB/c spleen cells cultured and labelled as described in the legend to Table 1. Group I, Con A present throughout, group II, Con A removed at 24 h. Anisomycin (10 ng ml^{-1}) and α -amanitin ($10 \mu\text{g ml}^{-1}$) were added to the culture medium as indicated. Each value represents the mean of four cultures.

mRNA with a $t_{1/2}$ of about 20 min (see ref. 10, in which actinomycin D, rather than α -amanitin, was used). If rRNA synthesis in lymphocytes is similarly affected by α -amanitin the effect must be reversible, as inhibition for 2 h is reversible (Table 2). It follows that the present results with α -amanitin cannot be attributed to an indirect effect on rRNA synthesis.

Using α -amanitin and anisomycin as inhibitors of mRNA transcription and translation respectively it has been shown that (1) the transformation factor in lymphocytes seems to be coded for by mRNA; (2) the mRNA has a $t_{1/2}$ of 3–4 h; (3) extracellular Con A is necessary for its continuous production when cell transformation is blocked by anisomycin. The effect of anisomycin is reversible and when mRNA translation is allowed to proceed normally (within 2 h of Con A removal), the cells transform independently of extracellular Con A. On the basis of these observations it is postulated that Con A induces the transcription of a new species of mRNA(s) in lymphocytes. When this mRNA is translated the newly synthesised protein takes over and commits the cells to enter the division cycle; extracellular Con A is no longer required.

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Release of vasoactive intestinal polypeptide in mast cells by histamine liberators

VASOACTIVE INTESTINAL POLYPEPTIDE (VIP), originally isolated from porcine duodenum¹, is a potent relaxant of vascular and nonvascular smooth muscle², and a stimulant of adenylate cyclase activity^{3,4}. The peptide has been localised in endocrine cells in the gastrointestinal tract and pancreatic islets^{5,6}, as well as in nervous structures in these and other organs^{7–9}, the peripheral autonomic nervous system and selected areas of the brain^{8–11}. We report here immunological evidence that VIP, or a closely related peptide, occurs in mast cells, from which it can be released by compound 48/80 or by the Ca^{2+} ionophore A23187.

Rat peritoneal mast cells were collected from the peritoneal cavities of rats. Under ether anaesthesia, the rats were injected intraperitoneally with 12 ml of Sorensen phosphate buffer, pH 7.0. The rats were killed with ether 5–10 min later, their abdomens were opened and the peritoneal fluid removed and centrifuged at 400g for 8 min. The cell pellet, resuspended in 2–3 ml of buffer per rat, contained approximately 10^6 mast cells (10% of the total cell population). Aliquots of washed cell suspensions were incubated for 15 min at 37°C with $0.1 \mu\text{g}$ per ml of compound 48/80 (Burroughs-Wellcome) or $1 \mu\text{g}$ per ml of Ca^{2+} ionophore A23187 (Lilly). After incubation the media were separated from the cells by centrifugation at 400g for 8 min. The supernatants were decanted and then assayed for VIP immunoreactivity by a specific radioimmunoassay¹² sensitive to 10 pg per ml, and for histamine by the fluorometric method of Shore¹³. The amounts of the peptide and amine released were expressed as per cent of their respective total

Table 1 VIP and histamine in rat peritoneal mast cells

	VIP	Histamine
Total content (ng ml) ⁻¹	3.6 ± 0.8 (6)	689.3 ± 3 (6)
Release (% of total)		
48/80	45 ± 10 (5)	48 ± 4 (5)
A23187	50 ± 10 (4)	67 ± 6 (4)

Figures are means ± s.e.m.: numbers of determinations are in parentheses.

content, measured after disrupting the cells by boiling in water for 3 min.

The mean VIP content in suspensions of rat peritoneal mast cells (3.6 ± 0.8 ng per ml or 1.4 ng per 10⁶ mast cells) was a fraction of their histamine content (689.3 ± 3 ng per ml); 1 ml contained approximately 4 × 10⁵ mast cells. Incubation of the cells with compound 48/80 or with A23187 provoked release of the peptide in proportions similar to those of the associated histamine release (Table 1).

For immunohistochemical studies, VIP antibodies were raised in rabbits against porcine VIP (GIH Laboratory, Stockholm), and included the same antibody (89N) used for radioimmunoassay, and two others (TR₁ and TR₂). Samples of lung and small intestine were obtained from adult and newborn Wistar rats and from mice, and were processed for immunohistochemistry according to the method of Pearse and Polak¹⁴. The samples were quenched in melting Arcton 22, freeze-dried, vapour-fixed in recrystallised benzoquinone at 60 °C for 3 h and embedded in paraffin; sections were cut at 5–7 µm. Pellets of rat peritoneal mast cells were processed for immunohistochemistry in the same way. The indirect immunofluorescence technique (24–48 h incubation at 4 °C) was carried out using the three VIP antibodies (dilutions 1:5 to 1:100), and antibodies against porcine secretin, synthetic human G-17 gastrin and porcine glucagon. As a second layer, fluorescein-labelled goat anti-rabbit IgG was used. The following control procedures were carried out: (1) incubation with VIP antisera (1:5) to which pure VIP (60 µmol per ml) had been added; (2) the first layer was replaced by non-immunised rabbit serum or phosphate buffer; (3) incubation with the second layer only. Following examination under the fluorescence microscope the sections were stained with toluidine blue (pH 5) to identify and confirm the presence of mast cells.

Sections of rat and mouse lungs incubated with VIP antisera (up to 1:100 dilutions) showed strong positive fluorescence in mast cells in perivascular, peribronchial and interstitial locations (Fig. 1). Examination at higher magnification revealed that VIP was localised within mast-cell granules (Fig. 1 inset). Mast cells

in sections of small intestine and in cell preparations from peritoneal lavage also showed strong VIP immunoreactivity. No fluorescence was observed with VIP-treated VIP antisera, or with the antisera against the other peptides. Mast cells were cross-identified in sections stained with toluidine blue; the cells contained typical metachromatic granules and their size, appearance and distribution were identical to those of VIP-immunoreactive cells.

The demonstration of immunoreactive VIP in mast cells from different locations from two mammalian species, and the release of the peptide by histamine releasers suggest interesting functional implications. The mast cell is the principal target cell in immediate hypersensitivity, playing a key part in anaphylaxis, extrinsic (allergic) asthma, hay fever and other IgE-dependent immunological responses¹⁵. The release of VIP by the ionophore A23187 is of special interest as this agent releases histamine by a mechanism similar to that of anaphylaxis¹⁶. As VIP stimulates the production of cyclic AMP in the lung and other tissues^{3,4}, and as agents which promote cyclic AMP accumulation in the lung also inhibit mediator release¹⁷, VIP could have an important modulating influence on the release of histamine and other mediators from mast cells.

Further, VIP may, when discharged from pulmonary mast cells, exert its relaxant actions on bronchial and vascular smooth muscle², providing another level of modulation of airway and vascular responses by the peptide.

VIP immunoreactivity has been reported in peptide extracts of normal porcine lungs¹⁸. The evidence presented here of VIP immunofluorescence in pulmonary mast cells provides an anatomical basis for that finding. Finally, mast cells are widely distributed in connective tissues of various organs of the body and are generally concentrated along the blood vessels. VIP in mast cells is therefore in a position to influence local or regional blood flow, in normal or pathological conditions.

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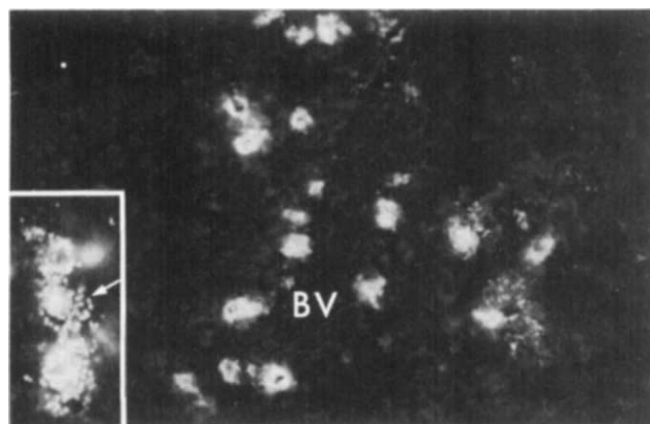
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Fig. 1 Many strongly fluorescent, VIP-immunoreactive mast cells in rat lung. Mast cells are scattered in the connective tissue, but some are concentrated along the wall of a blood vessel (BV). (Indirect immunofluorescence method, VIP antibody TR₂, 1:50, ×400.) Inset, higher magnification (×900) of mast cells showing VIP immunoreactivity confined to cytoplasmic granules (arrow).



Are dopamine receptors accessible to intracellularly applied agonist?

THERE is much evidence that receptors to neurotransmitters are generally on the outer surface of cell membranes¹⁻³. The experiments reported here raise the possibility that acinar cells of cockroach salivary glands may be activated intracellularly as well as by extracellularly applied agonists.

Studies were performed using the isolated innervated salivary gland of *Nauphoeta cinerea* (Olivier) bathed in flowing solution (2 ml min⁻¹) containing (mM) NaCl, 160; KCl, 1; CaCl₂, 5; Tris-HCl pH 7.6 buffer, 5 and mounted as previously described⁴. Cells were impaled with microelectrodes containing agonists in concentrations of up to 1.0 M dissolved in either potassium acetate or KCl. The electrodes were used not only for intracellular recording but also for ejecting the agonist by means of current pulses. The reservoir ducts were drawn into a small suction electrode to allow stimulation of the salivary nerve. Figure 1a shows two successive responses of an acinar cell, N to a single nerve stimulus and D to the application of a current pulse to the intracellular electrode equivalent to a charge of 40 nC. The responses can be seen to be very similar. In Fig. 1b the response to a burst of nerve stimuli, N, is followed by three responses to increasing ionophoretic charges and it can be seen that there is a graded relationship between amplitude of response and charge.

Intracellular applications of dopamine were made to about 100 cells and responses were obtained from about two-thirds of these. In a few experiments responses were also obtained from intracellular applications of adrenaline, noradrenaline and 5-hydroxytryptamine which, like dopamine, have also been shown to be effective when applied extracellularly⁵.

Two possible explanations for these responses are (1) receptors are activated intracellularly by the ionophoretically applied agonists or (2) the receptors on the outer membrane surface are activated by agonist which has leaked through the membrane. Attempts to distinguish between (1) and (2) have so far been inconclusive. In one experiment we compared the sensitivity of a series of cells to extra- and intracellular application from the same ionophoretic pipette. The cell was first impaled with a separate recording electrode, the ionophoretic pipette was brought as close as possible to the outer surface of the acinus and a response to a pulse of dopamine was elicited. An attempt was

Fig. 1 a, Lower trace, voltage; upper trace, current passed through recording electrode. N, intracellularly recorded response to a stimulus applied to the salivary nerve; D, response to a dopamine pulse ejected by a 100 nA current for 0.4 s. Downward deflection corresponds to hyperpolarisation. The electrode contained 0.7 M dopamine and 1 M potassium acetate. b, Intracellular response, N, to a burst of three nerve stimuli followed by responses to three pulses of dopamine ejected from the intracellular electrode by currents of 100 nA for durations of 0.2, 0.4 and 0.6 s respectively. The electrode contained 0.3 M dopamine and 1 M potassium acetate.

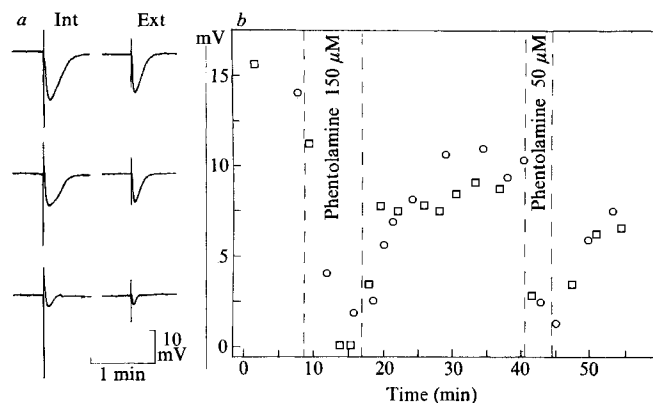
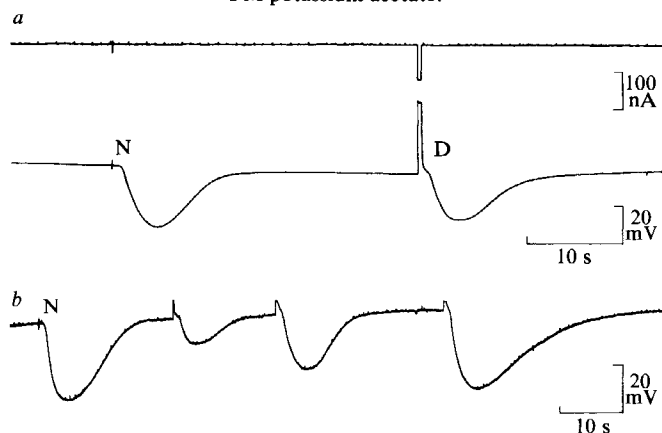


Fig. 2 Effect of phentolamine on intracellularly recorded responses from the same cell. a, Int, pulses of dopamine ejected from the intracellular electrode (100 nA for 0.4 s); Ext, pulses of dopamine ejected from an extracellular pipette (200 nA for 0.08 s). Both electrode and pipette contained 0.5 M dopamine and 3 M potassium acetate. Top records, control; middle records, during recovery from a short exposure to 150 μ M phentolamine; bottom records, in 25 μ M phentolamine. b, part of time course of experiment from which records in a were taken. $\circ$, Intracellularly applied dopamine; $\square$, extracellularly applied dopamine.

then made to insert the ionophoretic pipette. The initial recording electrode was usually dislodged in the process but this did not matter because if the cell was successfully impaled with the ionophoretic pipette it could also be used for recording. Little difference was found between the maximum sensitivities with the two different methods of application. For an electrode containing 50 mM dopamine in 3 M potassium acetate the largest value for the ratio of response to ionophoretic charge was 3 mV nC⁻¹ for intracellular and 2.3 mV nC⁻¹ for extracellular application. (If it is assumed that half the current is carried by cations and that the transport number of the dopamine ion is the same as that of K⁺, 1 nC would correspond to the ejection of about 10⁻¹⁶ mol; this would give rise to an internal concentration of about 10⁻⁷ M if the dopamine was uniformly distributed throughout an acinus of the usual diameter of about 100 μ m.) This result might be thought to argue against the leakage hypothesis, (2), but the result of a second type of experiment was found to be consistent with it. Cells were impaled with one ionophoretic pipette for intracellular recording and dopamine application and a second similar pipette was placed close to the acinar surface for extracellular dopamine application. After matching responses were obtained, phentolamine, which is known to antagonise extracellularly applied dopamine⁵, was added to the flowing bathing solution. Responses to both intracellular and extracellular dopamine were reduced to the same extent (Fig. 2); thus the leakage hypothesis cannot be rejected. However, as phentolamine may be able to leak into the acinar cells, the possibility that the receptors might be accessible from both sides of the membrane cannot be excluded.

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Ionophoretic model for Na-Ca counter transport

IN most mammalian cells the cytosolic concentration of free ionised Ca^{2+} is thought to approximate 10^{-6} – 10^{-7} M, whereas the extracellular Ca^{2+} concentration is close to 10^{-3} M (ref. 1). The efflux of Ca^{2+} across the cell membrane, which takes place against an electrochemical gradient, is apparently mediated either by a Ca^{2+} -stimulated ATPase² or by a Na-Ca counter transport process. In the latter process, the entry of Na^+ into the cell, which is a passive event driven by the electrochemical gradient of Na^+ across the plasma membrane, is thought to be coupled with the uphill outwards transport of Ca^{2+} (ref. 3). Although a possible modulatory effect of ATP on such a process has been suggested, it is generally assumed that the Na-Ca counter transport mechanism does not consume ATP (discussed in ref. 4). Because several passive ionic movements (such as the influx of Na^+ or Ca^{2+} into the cell) could be mediated by native ionophoretic molecules, we have explored whether it was possible, using an artificial system for the study of ionophoresis, to reproduce the major characteristics of the Na-Ca counter transport process. We report here our success.

A CCl_4 organic phase (3.2 ml) containing Br-X537A (1.4 mM, Roche) was placed in the bottom of a Pressman cell⁵. Ionophore Br-X537A was used because it transports both monovalent and divalent cations⁶. The composition of the supernatant aqueous phases (1.6 ml) which was placed in each chamber of the Pressman cell is given in the legend of Fig. 1.

At the end of the experiments—after 24 h—the immiscible organic phase contained only trace amounts of ^{22}Na , representing no more than $0.22 \pm 0.05\%$ of the total radioactivity in the system. The ^{45}Ca content of the final immiscible phase was greater. Relative to the total amount of radioactivity present in the system and with 4 mM Ca^{2+} in each chamber, it averaged $4.5 \pm 0.7\%$ ($n = 13$) and $11.8 \pm 1.0\%$ ($n = 8$) in the presence of Na^+ (121 mM in both chambers) and the absence of Na^+ , respectively. These values correspond to apparent calcium concentrations, in the immiscible phase of 0.18 ± 0.03 and 0.47 ± 0.04 mM (as distinct from 2.4 ± 0.9 μM in the absence of X537A).

In the absence of ionophore, no significant amount of ^{22}Na ever seemed to be translocated. Relative to the total amount of ^{45}Ca present in the system, the transfer rate for ^{45}Ca averaged no more than $0.21 \pm 0.03\%$ per h.

In the presence of Br-X537A but in the absence of ionic gradient, that is when both aqueous phases had the same ionic concentration at time zero, ^{45}Ca (which was added to only one of the chambers) moved towards the other chamber, and eventually the radioactivity was equally distributed among both chambers (Fig. 1, left). At the same Ca^{2+} concentration (4 mM), the initial rate at which the radioactivity moved from one chamber to the other was greater in the absence than in the presence of Na^+ (120 mM). The latter observation is consistent with the fact that Na^+ decreases the ability of the ionophore to translocate ^{45}Ca into an organic immiscible phase (our unpublished observation). When 4 mM Ca^{2+} and 120 mM Na^+ were present in both chambers, ^{22}Na was transferred relatively slowly (approximately 1.0% of the total radioactivity transferred per h) so that, even after 24 h, the partition equilibrium for ^{22}Na was not reached.

The pattern for ^{45}Ca translocation was different when an ionic gradient was imposed on the system at time zero. When 4 mM Ca^{2+} was present in both chambers, but Na^+ was distributed asymmetrically (120 mM out (O) and 6 mM in (I)), most of the ^{45}Ca initially added to the I compartment was transferred to the O compartment. After 24 h, more than 80% of the total radioactivity was recovered in the O chamber. This occurred independently of any electrical gradient, also being observed

when the two chambers were connected by an agar-agar bridge (4%, w/v, in Hartmann's solution Na^+ 158 mM, K^+ 5 mM, Ca^{2+} 2 mM, Cl^- 139 mM, $\text{CH}_3\text{-CHOH-CO}_2^-$ 28 mM). When, in addition to the Na^+ gradient (120 mM O and 6 mM I), a Ca^{2+} gradient (0.2 mM I and 4 mM O) was also introduced, up to 90% of the ^{45}Ca initially present in the I compartment was transferred to the O compartment. A dramatic uphill translocation of $^{45}\text{Ca}^{2+}$ (I $\rightarrow$ O) also occurred when only a trace of $^{45}\text{Ca}^{2+}$ (1.0 μM) and no $^{40}\text{Ca}^{2+}$ was added to the I chamber (data not shown). These observations indicate that ^{45}Ca can move against the Ca^{2+} gradient. The initial rate of ^{45}Ca transfer was much greater when the Ca^{2+} gradient was superimposed on to

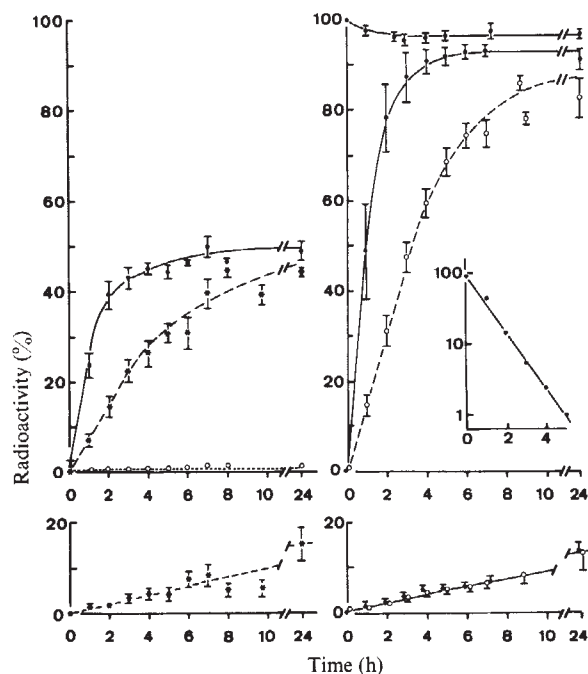


Fig. 1 Transfer of ^{45}Ca (upper graphs) and ^{22}Na (lower graphs) in a Pressman cell, across an immiscible organic phase containing Br-X537A. The aqueous phases consisted of a Hepes solution (25 mM) buffered to pH 7.0 with either NaOH or $\text{Ca}(\text{OH})_2$ and containing, as required, CaCl_2 , NaCl and trace amounts of $^{45}\text{CaCl}_2$ (0.5–1.0 $\mu\text{Ci ml}^{-1}$) or $^{22}\text{NaCl}$ (0.25 $\mu\text{Ci ml}^{-1}$). The organic phase was stirred continuously (about 120 r.p.m.) with a magnetic bar, the experiments being carried out at room temperature (about 22 °C). At the onset of the experiments and at timed intervals thereafter, a sample of the aqueous phase (0.05 ml) was removed from each chamber and examined for its radioactive content by liquid scintillation counting. The observed radioactivity was corrected for the change in volume due to the sampling procedure. Evaporation was minimised by the use of a glass cover and was corrected for by expressing individual data as a percentage of the total radioactivity recovered at each sampling time in both the I and O chambers. In the left panels, both aqueous phases contained Ca^{2+} (4 mM) in the presence (★) or absence (●) of Na^+ (120 mM). The control data for ^{45}Ca translocation obtained in the absence of Br-X537A are also shown (○). In the right-hand graphs, a Na^+ gradient (120 mM O, and 6 mM I) was always used, either the same Ca^{2+} concentration (4 mM) being initially present in both aqueous phases (○) or a Ca^{2+} gradient (0–0.2 mM I, and 4 mM O) being superimposed on the Na^+ gradient (●). The data for ^{45}Ca always refer to the radioactivity found in the O compartment, whether ^{45}Ca was initially located in the I (ascending curves) or O (upper flat curve) chamber. The data for ^{22}Na always refer to the radioactivity found in the I compartment, the tracer being in all cases initially located in the O chamber. In the inset (upper right graph), the data for ^{45}Ca transfer (●) are shown in a semi-logarithmic plot, the difference between equilibrium and observed values for ^{45}Ca being ranged on a logarithmic scale. Mean values ($\pm$ s.e.m.) refer to 6–12 individual experiments.

the Na^+ gradient than when only the Na^+ gradient was initially present (Fig. 1, right). This difference is attributable to the much lower initial Ca^{2+} concentration (0.2 mM compared with 4.0 mM) in the I compartment, so that much more radioactivity moved from I to O even if the same total Ca^{2+} was translocated in the same direction. Using a comparable experimental design (that is Na^+ 120 mM O and 6 mM I; Ca^{2+} nil I and 4 mM O) but with ^{45}Ca initially located in the O chamber, about 95% of radioactivity remained in the O chamber, even after 24 h of incubation. In all these experiments, ^{22}Na (which was initially located in the O chamber) moved in the I chamber at a rate comparable with that seen in the absence of Na^+ gradient (Fig. 1, lower graphs). Less than 15% of the total ^{22}Na was transferred from the O to the I chamber after 24 h of incubation.

The true concentration of Na^+ and Ca^{2+} in the two chambers was also measured. In the absence of ionophore, Na^+ and Ca^{2+} concentrations remained close to their respective initial values, whatever the experimental conditions (data not shown). In the presence of the ionophore Br-X537A and with a Na^+ gradient (Na^+ 120 mM O, and 6 mM I; Ca^{2+} 4 mM on both sides), the Ca^{2+} concentration progressively decreased in the I compartment and concomitantly increased in the O compartment at a mean rate of 0.48 ± 0.02 mM per h, so that 96% of the total amount of Ca^{2+} was present in the O chamber at the 8th h. In the presence of both Na^+ and Ca^{2+} gradient (Na^+ 120 mM O and 6 mM I; Ca^{2+} 4 mM O and nil I), no significant translocation of Ca^{2+} was observed, the concentration of Ca^{2+} plateauing at 4.06 ± 0.04 mM in the O chamber and no detectable amount of Ca^{2+} (less than 0.1 mM) appearing in the I chamber, even after 24 h. In both types of experiment, the Na^+ concentration slightly decreased in the O chamber and concomitantly increased in the I chamber at a rate not exceeding 0.8 ± 0.3 mM per h. Even at the 24th h, the concentration of Na^+ remained six to seven times higher in the O than in the I chamber.

The experimental condition which mimics more closely the situation encountered in most mammalian cells corresponds to the following distribution: in the O chamber (that is the extracellular fluid) Na^+ 120 mM and Ca^{2+} 4 mM; in the I chamber (that is the intracellular compartment) Na^+ 6 mM and $\text{Ca}^{2+} \leq 0.2$ mM. In such conditions the initial rate of transfer of radioactivity averaged 0.9% per h for $^{22}\text{Na}(\text{O} \rightarrow \text{I})$ and at least 88.0% per h for $^{45}\text{Ca}(\text{I} \rightarrow \text{O})$; see inset Fig. 1). These values correspond to cationic fluxes of 1.1 mM Na^+ per h and 0.2 mM Ca^{2+} per h. In living cells, three to four sodium ions are thought to enter the cell for each Ca^{2+} ion extruded by the Na-Ca counter transport process⁷.

In conclusion, Br-X537A can represent a model for the Na-Ca counter transport process. Our results show that a Na^+ gradient can be sufficient to maintain a Ca^{2+} gradient and to insure the transport of Ca^{2+} against such a gradient by ionophoresis.

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Potassium channels in the apical membrane of the toad gallbladder

It has been demonstrated that the apical and the basolateral cell membrane of the gallbladder have a high K^+ permeability^{1,2} and that the electrochemical potential for potassium in the cytosol is about 50 mV more positive than that in the bathing solutions³. Because of the existence of an $(\text{Na}^+, \text{K}^+)\text{ATPase}^4$ which is located on the basal lateral surface of the epithelial cells⁵, it is possible that K^+ is actively transported in the cells at the serosal side and partially leaves the cellular compartment through the apical membrane under the influence of its electrochemical gradient. In the present study we have investigated this hypothesis by analysis of the current fluctuations recorded in voltage-clamp conditions. This method has been shown to be very useful for the investigation of the kinetic properties of ion transport in such different biological tissues as nerve, postsynaptic membranes^{6,7} and epithelia⁸. We have found K^+ -selective channels which open and close randomly in the apical membrane of the toad gallbladder. However, this study did not reveal the presence of fluctuating ionic channels in the serosal membrane.

The gallbladder epithelium of the Mexican toad *Bufo marinus* was mounted in a modified Ussing chamber with a free surface of 0.16 cm². With Na Ringer's solutions (115 mM NaCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 1.08 mM K_2HPO_4 and 0.36 mM KH_2PO_4) on both sides, the transepithelial resistance was $212 \pm 15 \Omega \text{ cm}^2$ ($n = 79$) and the transepithelial potential was 0.44 ± 0.16 mV ($n = 34$), serosa positive. Strong amplification of this potential reveals that it is not constant but fluctuates around its macroscopic mean value. These voltage fluctuations were clamped to zero with a low-noise voltage-clamp circuit using a FET input amplifier⁹. The resultant current noise signal was sampled at a frequency of 500 Hz. The power spectrum of a time series of 2,048 data points was calculated on a PDP11/34 computer. The final spectra represent an average of 40 records.

With Na Ringer's on both sides, we observed a spontaneous lorentzian component in the power spectrum in 30 of 95 bladders (curve *a* in Fig. 1A). The plateau value (S_0) and the corner frequency (f_c) could be quite accurately determined in 16 bladders: $S_0 = 3.7 \pm 0.8 \times 10^{-19} \text{ A}^2 \text{ s cm}^{-2}$ and $f_c = 4.0 \pm 0.2$ Hz. In many cases, as in curve *a* of Fig. 1B, there was also a low-frequency noise component. In most of the experiments the lorentzian component was either masked by low-frequency noise or its intensity was too small to be detectable. The lorentzian noise component could be caused by one of the following mechanisms: (1) conductance fluctuations in one of the cell membranes or in the paracellular shunt produced by 'open and close' kinetics of ion-selective channels; (2) fluctuations in the active ion-transport flux in the case of an electrogenic transport mechanism. The relaxation time, τ , of the process we measured can be calculated from f_c ; thus, $\tau = 1/(2\pi f_c)$, being in this series of experiments 41 ± 2 ms. The addition of a metabolic inhibitor (3 mM KCN + 3 mM sodium iodoacetate) to the serosal solution resulted in a complete disappearance of the lorentzian component within 20 min (Fig. 1A). The application of 1 mM ouabain to the serosal solution caused a slow decrease of S_0 with a concomitant increase of f_c (Fig. 1B). From these experiments it cannot be concluded that the underlying transport mechanism is an active one because the inhibition of active transport reduces the electrochemical gradients across the mucosal and the serosal membrane². Consequently, it is possible that the metabolic inhibitors and ouabain cause an indirect decrease of S_0 by reducing the driving force for passive ionic movements. The increase of f_c after ouabain cannot be explained with the available data.

In most of the remaining bladders we investigated, large low-frequency components were recorded and a lorentzian component could not be detected with Na Ringer's on both

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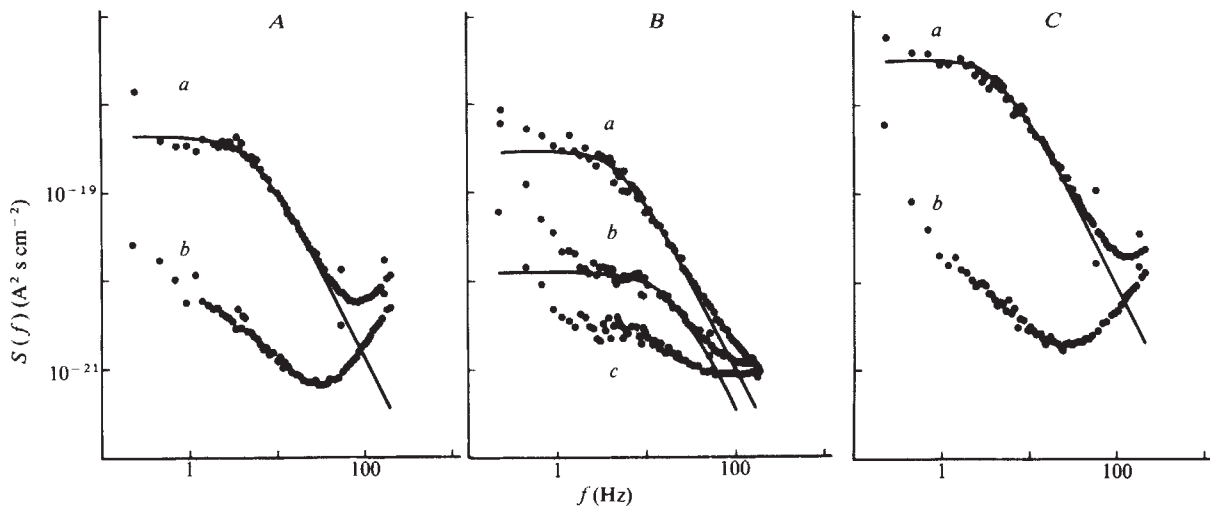


Fig. 1 Power spectra of the current fluctuations through the toad gallbladder epithelium in control conditions and after the addition of various agents. **A**, Curve *a*, spontaneous lorentzian noise spectrum recorded with Na Ringer's on both sides. The plateau value is $S_0 = 4.3 \times 10^{-19} \text{ A}^2 \text{ s cm}^{-2}$ and the corner frequency $f_c = 5.3 \text{ Hz}$. Curve *b*, fluctuations are depressed to levels comparable to the amplifier noise after the addition of 3 mM KCN+3 mM sodium iodoacetate. Because of the capacitive reactance component in the membrane impedance, the amplifier noise increases in the high frequency range and may become larger than the excess noise of the preparation¹⁵. This explains the increase of the spectral density observed at higher frequencies. **B**, Influence of ouabain. Curve *a*, spectrum recorded in control conditions: $S_0 = 2.7 \times 10^{-19} \text{ A}^2 \text{ s cm}^{-2}$ and $f_c = 5.7 \text{ Hz}$. Curve *b*, 15 min after the addition of 1 mM ouabain to the serosal solution: $S_0 = 1.2 \times 10^{-20} \text{ A}^2 \text{ s cm}^{-2}$ and $f_c = 17.1 \text{ Hz}$. Curve *c* was recorded 35 min after the addition of ouabain. **C**, Influence of TEA. Curve *a*, lorentzian recorded with Na Ringer's on the serosal side and with Ringer's in which all Na^+ was replaced by K^+ on the mucosal side: $S_0 = 3.1 \times 10^{-18} \text{ A}^2 \text{ s cm}^{-2}$ and $f_c = 4.7 \text{ Hz}$. Curve *b*, the addition of 5 mM TEA to the mucosal solution abolished the current fluctuations.

sides. The low-frequency component could be depressed by replacing 36 mM of the serosal Na^+ by K^+ . It cannot be excluded that these fluctuations, which we shall not discuss further, are due to smooth muscle activity, and consequently can be depressed by a K^+ -induced depolarisation. A lorentzian could be shown in 71% of these preparations after replacing all Na^+ by K^+ at either the mucosal or the serosal side. This indicates that the underlying mechanism depends on the presence of potassium in the bathing solutions. We therefore investigated the dependence of S_0 and f_c on the mucosal ($[\text{K}]_m$) and the serosal ($[\text{K}]_s$) K^+ concentration. These experiments were done with bladders which showed a spontaneous lorentzian component in the power spectrum. During all Na^+ - K^+ replacements f_c did not change significantly. The substitution of 36 mM of the mucosal Na^+ by K^+ led to a complete disappearance of the lorentzian component (Fig. 2). The increase of $[\text{K}]_m$ reduces the electrochemical gradient across the mucosal membrane¹¹, so that possibly the K^+ efflux to the mucosal side decreases. A further increase of $[\text{K}]_m$ enhanced the fluctuations, and the lorentzian component reappeared. At $[\text{K}]_m = 115 \text{ mM}$, S_0 was even larger than in control conditions. In these circumstances a K^+ influx through the mucosal membrane can be expected. An increase of $[\text{K}]_s$ led to an increase of S_0 over the entire range of K^+ concentrations. Even after poisoning the epithelium with a metabolic inhibitor, the lorentzian component could be observed in the spectra when a large K^+ gradient was applied.

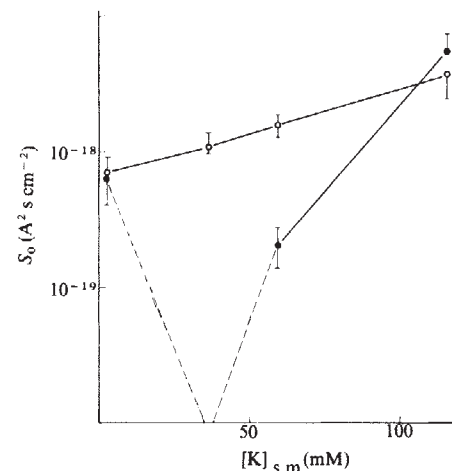
From these data it can be concluded that the relaxation process underlying the lorentzian component is related to K^+ movements through the mucosal membrane. The gradual rise of S_0 with $[\text{K}]_s$ can be understood because the increase of $[\text{K}]_s$ depolarises the intracellular potential² and possibly leads to an increase of the cellular K^+ concentration. Consequently, the increased electrochemical gradient for K^+ across the mucosal membrane results in a rise in the K^+ efflux.

More evidence for the existence of K^+ -selective channels in the mucosal membrane was found in the following experiments. It is known that tetraethylammonium (TEA) blocks the K^+ channels in nerve membrane¹² and reduces the permeability of the mucosal membrane of the gallbladder¹. The addition of 5 mM TEA to the mucosal bathing solution completely

inhibited the lorentzian noise components (Fig. 1C). This was observed for the spontaneous lorentzian current fluctuations recorded with Na Ringer's on both sides, as well as in experiments where the lorentzian was produced by a K^+ gradient across the epithelium. The inhibitory effect of TEA occurred in less than 5 min and was completely reversible. Added to the serosal side, TEA had no effect on the fluctuations. This clearly demonstrates that the fluctuating K^+ channels are located on the mucosal side.

As the paracellular shunt path of the gallbladder has a high conductance¹³, especially for K^+ (ref. 2), we investigated the possibility that the fluctuations are caused by a K^+ flux through

Fig. 2 Plateau values, S_0 , as a function of the serosal ($[\text{K}]_s$) and mucosal ($[\text{K}]_m$) K^+ concentration. Na^+ was replaced by K^+ at constant ionic strength. Closed circles represent the K^+ replacements at the mucosal side with normal Ringer's on the serosal side. The open circles are data obtained with normal Ringer's on the mucosal side and with different $[\text{K}]_s$ values. At $[\text{K}]_m = 36 \text{ mM}$, S_0 was smaller than the amplifier noise level, which is about $1.0 \times 10^{-21} \text{ A}^2 \text{ s cm}^{-2}$. As the exact position of this point is unknown, it is indicated with dashed lines, s.e.m. values are indicated.



the shunt. The cationic permeability of the paracellular pathway can be decreased with the addition of 20 mM 2,4,6-triaminopyrimidine (TAP) to both sides of the epithelium¹⁴. With Na Ringer's on both sides, the transepithelial resistance increased in this series of experiments from 128 ± 20 to $355 \pm 38 \Omega \text{ cm}^2$ ($P < 0.0005$, $n = 8$). When all Na^+ was replaced on both sides by K^+ , an increase from 169 ± 46 to $251 \pm 67 \Omega \text{ cm}^2$ ($P < 0.01$, $n = 6$) was found. We therefore assume that TAP reduces the K^+ permeability of the paracellular shunt, as was shown for other cations¹⁴. The plateau values of the lorentzians recorded either with Na Ringer's on both sides, or in the presence of a K^+ gradient across the epithelium, did not change significantly with the addition of 20 mM TAP to both bathing solutions. Therefore, as TAP reduces the paracellular K^+ fluxes and does not change the lorentzian current fluctuations, it seems unlikely that the K^+ -selective structures are located in the paracellular pathway.

From our data, we conclude that K^+ -selective channels, which open and close randomly, exist in the mucosal membrane of the toad gallbladder. No rectification characteristics could be shown, as both an inward- and an outward-directed K^+ gradient across the mucosal membrane caused an increase of the lorentzian current fluctuations. TEA, as in other tissues, acts as a reversible blocker for the K^+ channels. Further investigations, which allow us to determine the probabilities for the open and closed state, can give an insight into the mechanism which controls the K^+ permeability.

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Absence of measles proviral DNA in systemic lupus erythematosus

MEASLES VIRUS usually causes an acute self-limiting infection in humans and a productive cytolytic infection in susceptible tissue culture cells. However, measles-like virus has been isolated from the brains of patients with subacute sclerosing panencephalitis (SSPE), a chronic progressive neurological disorder of children¹. Also, persistent measles infection has been implicated in the pathogenesis of several chronic diseases of unknown aetiology, including multiple sclerosis² and systemic lupus erythematosus (SLE)³. Manipulation of the conditions of infection can result in persistently infected tissue culture cells, which have been proposed as a model system for studying the molecular mechanisms responsible for cytolytic virus persistence in man^{4–6}. There is experimental evidence for at least four mechanisms leading to the induction of persistent measles infections: the production of defective virus particles which interfere with the replication of complete virus⁷, the generation of mutant virus strains with the capacity for non-cytolytic replication⁸, and infection of certain cell types (for example, neurones) restricted in their capacity to support all steps in virus

replication⁹. Zhdanov *et al.*^{10–13} have proposed that measles virus persists in cells by a mechanism similar to that shown for retroviruses, that is, the synthesis of a DNA copy of the viral genome and the insertion of the proviral DNA into the host cell chromosome. This would be a novel mode of replication, as the replication of measles virus in cytolytic infections involves a double-stranded RNA intermediate. Using tritium-labelled viral RNA probes in molecular hybridisation assays, Zhdanov *et al.* have demonstrated the presence of measles virus-related DNA in chick embryo cells¹¹ and human lymphoid cells¹³ persistently infected with measles virus. Further, they detected measles virus-related DNA in leukocytes, lymph nodes, kidney and urine cell sediment from five SLE patients¹². Negative results were obtained using cells from appropriate control tissues. The obligative reverse transcriptase was found in the persistently infected cell lines and SLE tissues, and concomitant retrovirus infection was proposed as a prerequisite for persistent measles infection. Thus, these studies not only provide insights into the molecular mechanisms of measles virus persistence but suggest aetiological relationships for a fatal disease of unknown cause. However, we report here that, using a more sensitive nucleic acid hybridisation assay, we were unable to confirm the presence of measles virus-related DNA in either persistently infected cells or cells from patients with SLE.

We used a radioisotopically labelled complementary DNA probe (MV cDNA) generated from an *in vitro* reverse transcriptase reaction, using measles virus-specific mRNA as template and reverse transcriptase from avian myeloblastosis virus. Reaction conditions were selected to optimise the extent of transcription of the viral mRNA^{14,15}. The measles virus-specific mRNA (MV mRNA) used as template in this reaction was obtained by infecting CV-1 cells at a high multiplicity of infection and then treating the cells with actinomycin D and cycloheximide (for details see legend to Fig. 1). High concentrations of actinomycin D ($25 \mu\text{g ml}^{-1}$) are necessary for complete suppression of host cell RNA synthesis in infected cells¹⁶. Cycloheximide decreases virion RNA synthesis but increases the yield of viral mRNA¹⁷. Infected cells were ruptured and polyribosomes isolated by differential centrifugation. RNA was extracted from polyribosomes by caesium chloride centrifugation and poly-A-rich RNA purified by affinity chromatography. MV mRNA had a sedimentation coefficient of $\sim 20\text{S}$ in sucrose–SDS gradients and its separation into discrete species by formamide–polyacrylamide gel electrophoresis is reported elsewhere¹⁸.

MV ³H-cDNA was purified as described in the legend to Fig. 1. The cDNA was 93% single stranded, as determined by sensitivity to S_1 nuclease, and had a sedimentation coefficient of $\sim 7\text{S}$ in alkaline sucrose gradients¹⁹. Hybridisation of ³²P-labelled MV mRNA to excess MV ³H-cDNA (ratio 1:10) protected 90% of the labelled RNA from hydrolysis with RNase¹⁸. Further evidence of nearly complete transcription of the RNA template was found in the kinetics of annealing of the cDNA to the MV mRNA template (Fig. 1). The half-saturation value ($C_{0.5}$) of the MV probe to its template was compared with the $C_{0.5}$ of polio cDNA annealed to polio virion RNA using the relationship described by Birnstiel *et al.*²⁰. A nearly complete DNA copy of polio RNA (98% of polio genome) was synthesised using the method of Kacian and Myers¹⁷. The kinetic complexity of MV mRNA was 2.3-fold greater than that of polio RNA. As the molecular weight of polio RNA is estimated to be $\sim 2.6 \times 10^6$ (ref. 21), the calculated complexity of MV mRNA of $\sim 6 \times 10^6$ is consistent with data obtained from sedimentation analysis of virion RNA²². The thermal stability of the measles virus mRNA–cDNA hybrid was analysed by hydroxyapatite chromatography; the T_m value was 87.5°C (temperature at which 50% of hybrid eluted from the column), indicating a high degree of base-pair matching²⁵.

The specificity of the viral cDNA probe was demonstrated by a series of hybridisation experiments shown in Table 1. There was minimal hybridisation to relevant synthetic homopolymers (rA, rG) and unrelated viral and bacterial RNA, and a small

degree of hybridisation to monkey kidney cell nucleic acid (<6%). It is likely that this results from *in vitro* transcription of normal cell mRNA contaminating the MV mRNA preparation used as template. If there is non-preferential transcription of viral and cell mRNA, the percentage of the cDNA hybridising to normal cell DNA represents the fraction of the template which is cell mRNA. This fraction has varied over ~3–20% using probes generated from different MV mRNA preparations. 87% of the probe hybridised to RNA from infected cells but there was only minimal hybridisation to DNA from either lytically or persistently infected cells. A higher degree of hybridisation to RNA from lytically infected cells probably did not occur because of the presence of unlabelled negative-strand (virion) RNA which competes with the probe for positive-strand viral mRNA in the hybridisation reaction. Removal of negative-strand viral RNA from the RNA preparation by oligo-dT cellulose chromatography increases the hybridisation of the probe to 94%.

We attempted to hybridise the MV cDNA probe to DNA from various organs (spleen, kidney, placenta) of 15 patients with SLE, and DNA from six normal individuals (that is, the organ demonstrated no histopathology). With the exception of four patients from whom placenta was obtained, all patients had aggressive disease and the majority of specimens were obtained at necropsy. The conditions of the annealing reactions were as described in the legend to Fig. 1. Annealing mixtures contained 210 µg of cell DNA and 400 pg of ³H-cDNA, and were incubated to a C_{ot} value of 5,000. We found that <5.2% of the probe hybridised to the DNA samples from SLE patients compared with 1.0–5.2% hybridisation to DNA from the same organs of normal controls. Similarly, there was no difference in cDNA–cell RNA hybridisation experiments using RNA from seven SLE patients and four normal controls in the same reaction conditions as those used in the DNA–DNA hybridisation reaction. 1.0–2.5% of the probe hybridised to RNA from SLE samples compared with 2.5–3.5% hybridisation to RNA from normal controls.

Therefore, the data described here do not support the findings of Zhdanov *et al.*^{10–13}. We were unable to find evidence of measles virus-related DNA sequences in persistently infected cell lines despite the use of a nucleic acid probe with 2 logs higher specific activity than that used in their studies. The persistently infected cell lines studied by Zhdanov *et al.* showed evidence of concomitant infection with a retrovirus, as determined by the presence of 70S RNA and reverse transcriptase. We could not detect evidence of retrovirus infection in our cell lines by assays

for viral reverse transcriptase²⁸, despite previous reports that some clones of HEp-2 cells contained type 'B' retrovirus²⁹. Differences in the expression of this retrovirus-specific enzyme by the cell line could determine whether other viral RNA molecules are transcribed into a DNA form and integrated.

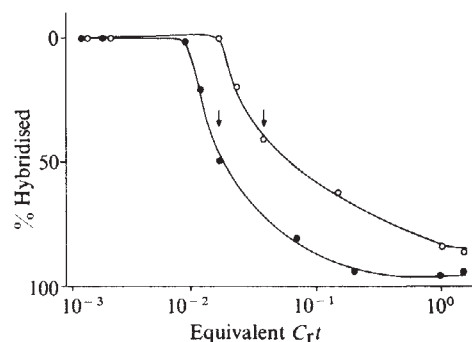


Fig. 1 Hybridisation of viral RNAs to ³H-cDNA probes. MV mRNA (○) and polio virion RNA (●) were hybridised to their respective cDNA. CV-1 cells grown in monolayer in minimum essential media with 5% fetal calf serum (MEM-FCS) were infected with the Edmonston strain of measles virus at an MOI (multiplicity of infection) of 10, for 2 h at 37 °C. Four days after infection, media were replaced with fresh MEM containing 25 µg ml⁻¹ actinomycin D (Sigma). Two hours later cycloheximide (Sigma) was added to a final concentration of 75 µg ml⁻¹. Six hours after the addition of cycloheximide, the cells were removed from the flask with trypsin and centrifuged for 10 min at 200g. They were resuspended in a solution containing 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM MgCl₂ and 5% sucrose (TNM), and kept at 4 °C for 15 min. The cells were broken by 5–20 strokes of a tight glass Dounce homogeniser and cell disruption monitored by phase contrast microscopy. Nuclei were centrifuged for 10 min at 600g and the nuclear pellet washed once more with the hypotonic buffer. The post-nuclear pellet supernatants were pooled and layered on a discontinuous gradient containing 13.4 ml of 1 M sucrose (in TNM with 100 µg ml⁻¹ polyvinyl sulphate) over 4 ml of 2.5 M sucrose in an SW27 rotor and centrifuged at 27,000 r.p.m. for 3 h at 4 °C. The opalescent polyribosomal band at the interface of the two sucrose layers was removed by puncturing the side of the tube. The polyribosomal sample was then made to 0.5% SDS and 1 M mercaptoethanol and RNA extracted by the method of Glisin *et al.*²⁴. CsCl was added at 1 g per ml of sample, and the mixture shaken for 15 min, then layered on 1.5 ml of 5.7 M CsCl (in 0.1 mM EDTA) and centrifuged at 35,000 r.p.m. for 12 h in an SW50.1 rotor at 20 °C. The RNA pellet was resuspended in 0.01 M Tris-HCl, pH 7.5, 0.5 M NaCl and poly-A-rich RNA obtained by passing the sample twice through an oligo-dT cellulose column¹⁸. The eluate from the column was pooled, made to 0.4 M NaCl, precipitated with two volumes of ethanol, and the RNA used as template in a reverse transcriptase reaction. The total reaction (0.1 ml volume) contained 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 8 mM MgCl₂, 0.5 mM dithiothreitol, 1 mM each of dATP, dTTP, dGTP and ³H-dCTP (29.3 Ci mmol⁻¹, New England Nuclear), 100 µg ml⁻¹ actinomycin D, 5 µg MV mRNA, 1 µg dT₁₀ and 5 units avian myeloblastosis reverse transcriptase. For poliovirus RNA templated reactions, 35S polio RNA was used¹⁵. The reaction was incubated for 1 h at 37 °C and terminated by the addition of 0.1 M EDTA. RNA was hydrolysed and ³H-cDNA purified by methods reported previously²⁵. The specific activity of the ³H-cDNA was calculated to be ~10⁷ c.p.m. per µg DNA. Hybridisation reactions were carried out in 20–40-µl volumes in glass capillaries. The reaction mixture contained 50 mM Tris-HCl, pH 7.0, 0.5 M NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), 0.2 µg of viral RNA (or total *E. coli* RNA) and 4,000 c.p.m. of ³H-cDNA probe. The polio cDNA was sheared to a size comparable with that of the MV cDNA because of the effect of single-strand length on kinetic complexity³¹. Samples were heated to 100 °C for 2 min and incubated at 65 °C. Equivalent C_{ot} (product of RNA concentration, time of incubation and factor for cation concentration) was determined by the method of Britten and Kohne²⁶. S₁ nuclease analysis of the hybridisation reaction was by the method of Vogt²⁷.

Table 1 Hybridisation of MV ³H-cDNA to various nucleic acids

RNA	% Hybridised
Poly rA	<1%
Poly rG	<1%
Avian myeloblastosis virus	<1%
Newcastle disease virus	<1%
<i>Escherichia coli</i>	<1%
CV-1 cells	3.6%
CV-1 cells, MV-infected	87.0%
DNA	
<i>E. coli</i>	<1%
CV-1 cells	5.7%
CV-1 cells, MV-infected	4.6%
Hamster embryo cells, MV-infected	4.0%
HEp-2 cells, MV-infected	5.2%

The hamster embryo cell line⁶ and HEp-2 cell line³⁰ were persistently infected with MV. Both cell lines produced 10²–10³ plaque-forming units per ml per 24 h and >90% of cells haemadsorbed rhesus-monkey erythrocytes. Hybridisations were carried out as described in the legend to Fig. 1, and reactions with viral RNA were taken to a C_{ot} = 1 and those with cell RNA and DNA to a C_{ot} = 5,000 (ref. 26). DNA was extracted by a method described previously²⁵ and RNA extracted by the method of Glisin²⁴.

Also, we were unable to confirm the presence of measles virus DNA or RNA nucleotide sequences in cells from patients with SLE. Alekberova *et al.*¹¹ detected 2–5 measles virus gene equivalents per cell, well within the limits of sensitivity of our assay. In reconstruction experiments, we could unequivocally detect 1 viral gene equivalent per cell. One could construct an hypothesis for a viral cause of SLE in which few cells were infected and infected cells had a low level of viral gene expression. In this situation, viral information would escape detection by the hybridisation assays carried out in the present study. However, our findings, plus evidence of the non-viral nature of the tubulo-reticular structures in SLE and the nonspecificity of increased antiviral antibody titres in this disease³, indicate that it is unlikely that measles virus has a role in the pathogenesis of SLE. Collaborative studies which are now in progress through the USA/USSR Scientific Exchange programmes should clarify the discrepancy between the results reported here and those of Soviet investigators. Further, this study has shown that viral cDNA probes can be used to examine molecular aspects of measles virus replication and persistence, and may be useful in clarifying the role of measles virus in other human diseases, particularly multiple sclerosis.

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Expression of endogenous C-type viral antigen on normal mouse haemopoietic stem cells

PARAMETERS such as size, density and differences in cell-cycle kinetics have been used to distinguish murine pluripotent haemopoietic stem cells (colony-forming units in the spleen, CFU-S) and committed haemopoietic progenitor cells¹. Efforts to use surface membrane markers^{2–4} have been based on two different, mutually exclusive concepts that regard pluripotent stem cells either as 'null-cells' or 'multiple-marked' cells. The first concept suggests that the markers are sequentially expressed during differentiation and maturation of a cell. Pluripotent stem cells, being the most undifferentiated haemopoietic cells, would be expected to have no markers or at least very few. According to the 'multiple-marked' stem cell hypothesis, however, differentiation would be accompanied by a specific loss of markers⁵. CFU-S can be detected by the development of spleen colonies after transplantation of normal syngeneic bone marrow cells (BMC) into irradiated recipients⁶. Committed granulocyte-macrophage colony-forming cells (GM-CFC) and early or late erythroid (BFU-E or CFU-E) progenitor cells can proliferate clonally in semisolid medium *in vitro* if stimulated by specific growth factors, namely granulocyte-macrophage colony-stimulating factor (GM-CSF) or erythropoietin^{7–9}. Pretreatment of normal marrow cells with a rabbit antiserum against mouse brain tissue has been reported to suppress spleen colony formation *in vivo* without influencing the growth of granulocyte-macrophage colonies *in vitro*¹⁰. We now report that antigens coded by endogenous C-type virus are expressed on pluripotent stem cells but were not detected on committed progenitor cells.

Our approach was suggested by recent experiments where an antiserum directed against endogenous xenotropic C-type virus suppressed the humoral immune response in all mouse strains tested^{11,12}. As immunocompetent cells are ultimately derived from pluripotent stem cells of the lymphoid and myeloid system¹³, it is conceivable that viral antigens are also expressed on cells of the haemopoietic system where they may have a physiological role, as suggested for cells participating in the immune response.

Fig. 1 Effect of preabsorption with virus on capacity of antiserum to suppress colony formation by haemopoietic stem cells. Marrow cells were incubated with medium or serum as described in the legend to Table 1. The final dilution of the serum was 1:80. 400 µg of density-gradient-purified virus was pelleted by centrifugation, incubated with 50 µl of serum diluted 1:10 for 60 min in ice and removed again by centrifugation. *a*, Medium control; *b*, xenotropic BALB/c virus absorbed antiserum. LPS-induced xenotropic BALB/c virus used for absorption was grown in mink cells (CCL-64); *c*, Friend leukaemia virus-absorbed antiserum; *d*, tobacco mosaic virus-absorbed antiserum; *e*, non-absorbed antiserum.

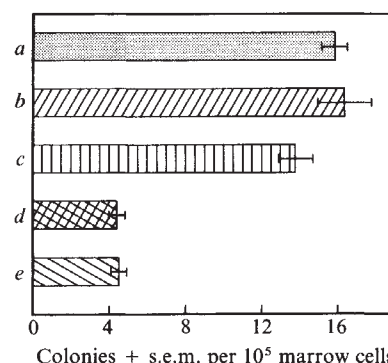


Table 1 Effect of pretreatment of marrow cells with anti-xenotropic virus serum on the number of CFU-S and GM-CFC

Serum (final dilution)	CFU-S		GM-CFC	
	Colonies ± s.e.m. per 10 ⁵ BMC	% inhibition of CFU-S	per 10 ⁵ BMC no C'	+C'
Control serum				
1:5	14.3 ± 1.2	7.6	146	234
1:20	ND	—	112	200
1:80	ND	—	124	215
1:320	ND	—	124	186
Antiserum				
1:5	0.4 ± 0.2	97.6	224	200
1:20	0.2 ± 0.1	98.6	164	212
1:80	0.2 ± 0.1	98.7	96	215
1:320	6.9 ± 0.7	55.1	164	192
No serum	15.5 ± 1.3	—	86	114

Marrow cells were flushed from the femurs of 12-week-old male SPF C57BL/6 mice with 1 ml RPMI 1640 medium (Microbiological Associates), supplemented with antibiotics and 0.02 M HEPES. Influence of antiserum on CFU-S: 2×10^6 viable nucleated cells were incubated at different dilutions of serum without complement (C') in a final volume of 0.4 ml at 0 °C. 30 min later the cells were diluted 1:25 with supplemented RPMI 1640 and washed. The cells were then injected intravenously into syngeneic mice pre-irradiated with 800 rad (10^5 viable nucleated cells per mouse, 15 mice per group). The spleens of the mice were removed 9 d later, fixed for 10 min in Bouin's fixative and the number of macroscopic colonies counted⁶. Influence of antiserum on GM-CFC: 3.2×10^5 viable nucleated cells were incubated with different dilutions of serum with or without complement in a final volume of 0.16 ml for 45 min at 37 °C and 7.5% CO₂. After three washings in the cold (5 min, 200g), aliquots of 1×10^5 cells were cultured in Petri dishes containing 1 ml of Dulbecco's modified Eagle's medium (Gibco) supplemented with antibiotics, and an amino acid-vitamin cocktail¹⁶ 15% horse serum (Microbiological Associates), 0.8% methylcellulose¹⁷ (Dow) and 5% mouse-lung-conditioned medium¹⁸ (representing a plateau concentration of GM-CSF) at 37 °C and 10% CO₂ in a humidified incubator. Colonies containing more than 50 cells were counted 7 d later. Each number represents the mean value of duplicate cultures. ND, not determined.

The antiserum used was from the same pool previously reported to be immunosuppressive^{11,12} and was prepared against BALB/c xenotropic endogenous C-type virus originally induced from spleen cell cultures by treatment with bacterial lipopolysaccharide (LPS)¹⁹ and collected in stably infected rabbit cornea (SIRC) cells.

To examine the effect of the antiserum on mouse CFU-S, normal marrow cells were incubated either with antiserum or control serum *in vitro*, washed and then injected into irradiated recipients. No exogenous complement was added, as it was assumed to be present in the recipients. A typical experiment (Table 1) showed that the development of spleen colonies was suppressed by pretreatment of the donor cells with antiserum. Suppression was not always complete, but with high concentrations of antiserum it was never less than 75%. In other experiments, marrow cells were incubated with antiserum and complement. After washing, cells were assayed for GM-CFC (Table 1). The antiserum did not inhibit colony development whether or not complement had been added.

In other experiments BFU-E and CFU-E frequencies were determined in marrow cells preincubated in identical conditions with combinations of antiserum, control serum and complement. Again, no influence could be detected (L. Guilbert and F.G.S., unpublished data). However, some experiments (data not shown) indicate that preincubation of spleen cells with antiserum and complement can efficiently suppress the frequency of B-lymphocyte colony-forming cells (BL-CFC). The failure of antiserum plus complement to reduce the number of colonies derived from GM-CFC or erythroid precursor cells does not definitely prove that these cells lack the corresponding antigen. The failure could also reflect low antigen density and consequent low antibody concentration on the cell-surface

insufficient to initiate complement activation. To clarify this, marrow cells were incubated with antiserum in the cold and subsequently with a goat anti-rabbit immunoglobulin (GARIG) and complement in the warm. The ability of the anti-immunoglobulin to bind complement, the lytic activity of the particular complement batch used and the efficiency of the procedure to detect low concentrations of cell-surface-bound antibody unable by itself to activate complement were tested in parallel in another system. Mouse thymus cells preincubated with a non-lytic dilution of a rabbit anti-mouse thymus cell serum were readily killed by a second incubation with GARIG plus complement. However, GM-CFC were not reduced by this procedure (Table 2). These data strongly suggest that GM-CFC do not bind significant amounts of antiviral antibodies, although

Table 2 Failure to detect endogenous viral antigen on GM-CFC by indirect cytotoxicity

Treatment of cells pre-sensitised with antiserum		Results	
GARIG (final dilution)	C'	BMC + anti-xenotropic virus serum (GM-CFC per 10 ⁵ BMC)	Thymus cells + antithymus cell serum (% cytotoxicity)
—	—	101	4
—	+	81	15
1:80	—	78	5
1:80	+	92	96

Indirect cytotoxicity for GM-CFC: marrow cells were prepared and handled during the first step as described in the legend to Table 1, but incubated with a 1:20 dilution of anti-xenotropic virus serum for 30 min at 0 °C. The cells were then washed twice (5 min, 200g) in the cold and incubated for 45 min at 37 °C and 7.5% CO₂ in air with GARIG (Behringwerke) and complement. After two further washings with the medium, the cells were assayed for GM-CFC as described in the legend to Table 1. Each number is the mean value of duplicate cultures. Indirect cytotoxicity test for thymus cells: C57BL/6 thymus cells were prepared by standard techniques in RPMI 1640 supplemented with 2% FCS. Aliquots of 5×10^5 cells were incubated with rabbit anti-mouse thymus cell serum in a volume of 0.05 ml and a final dilution of 1:2,560 for 30 min at 0 °C in microtitre plates. 30 min later 0.1 ml of medium was added and the cells washed twice in the cold. The cells were resuspended in 0.05 ml of medium containing GARIG and complement. After a second incubation for 45 min at 37 °C, the percentage of killed cells was determined by Trypan blue dye exclusion. A minimum of 200 cells were counted per sample.

an extreme resistance to complement-mediated cytolysis cannot definitely be excluded. Support for the view that the antiserum does not react with GM-CFC was obtained when the IgG fraction of the antiserum was added directly to the culture dishes. This procedure did not influence GM-CFC but it effectively inhibited the growth of BL-CFC (data not shown).

To prove that the inhibitory effect of the antiserum on spleen colony formation was due to its antiviral specificity, absorption experiments were carried out with equal amounts of different viruses. To exclude co-absorption of possible reactivities against rabbit cell-membrane antigens, xenotropic C-type virus used for absorption was grown in mink rather than rabbit cells, and was found to neutralise the activity of the antiserum completely. Preabsorption with Friend virus was almost as effective. This was expected, as Friend virus also neutralised the immunosuppressive activity of this serum¹². Crossreactivity between Friend and BALB/c xenotropic virus is consistent with the claim that the Friend virus genome contains sequences derived from endogenous xenotropic virus¹⁴. On the other hand, tobacco mosaic virus, an unrelated virus, had no ability to reduce anti-stem-cell activity of the serum (Fig. 1).

The data presented here show that an antiserum against endogenous C-type virus suppresses the development of spleen colonies generated by pluripotent haemopoietic stem cells. On the other hand, the antiserum displayed no detectable effect on GM-CFC and erythroid precursor cells. The most likely explanation for the observed effect is that stem cells express viral antigen and are killed *in vivo* by a mechanism involving the Fc portion of bound antibodies and possibly complement. It is tempting to speculate that endogenous viral antigens are physiologically required for the differentiation of haemopoietic cells in a similar way to that suggested for cells differentiating during an immune response^{11,12,15}. It is interesting that (Fab')₂ fragments of the antibody used have been found to suppress an *in vitro* immune response demonstrating complement independence of the phenomenon (C.M. and G. Schumann, unpublished results). This serum may be useful for understanding haemopoiesis.

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Certain rheumatoid factors react with nucleosomes

NATURAL antibodies against antigenic sites on the Fc region of human and animal IgG are referred to collectively as rheumatoid factors (RF) and they represent one of the immunological hallmarks of rheumatoid arthritis (RA). Thus, the synovial fluid and tissue of RA patients contain large immune complexes which at least in part consist of RF^{1–4}, and RF are present in serum in the majority of patients with definite or classical RA. Recent studies have demonstrated that purified and pepsin-digested polyclonal RF of IgG class from an RA serum (Han), in addition to reacting specifically with IgG, also bind to an antigen associated with cell nuclei from many species⁵. Furthermore, essentially 100% of the anti-nuclear antibodies (ANA) of two sera (Han, Jos) was absorbed by insolubilised human IgG; the

antibodies which could be eluted from this immunosorbant bound to cell nuclei, and this binding was specifically inhibited by Fc fragments of human IgG^{5,6}. We show here that RF from serum Jos bind to nucleosomes, the repeating histone–DNA subunit of chromatin.

Mono-, oligo- and polynucleosomes were prepared by nuclease digestion of chicken erythrocyte nuclei (see legend to Fig. 1). Chicken red cells were chosen because contamination with immunoglobulins (Ig) could be avoided by washing. Nucleosomes in the nuclear digest were fractionated into two major pools by gel filtration on Sepharose 4B (Fig. 1). Pool 1 and the 150 mM NaCl-insoluble fraction of pool 2 contained all the major histone classes (Fig. 1b, d) and essentially no non-histone proteins (Fig. 1e, f). DNA from pool 1 formed a series of bands consistent with the presence of multiples of the 200 base pair monomer DNA (Fig. 1g). The size of the DNA from pool 2 corresponded roughly to nucleosome monomers and dimers (Fig. 1i). Sepharose 4B gel filtration in 0.6 M NaCl stripped the chromatin of H1 and H5, but H2a, H2b, H3 and H4 were still present (Fig. 1c).

The nucleosomes of pools 1 and 2 were tested for their ability to absorb RF specifically. Serum Jos, which contains RF with high avidity for cell nuclei⁶, and serum Ter whose RF lack ANA activity, were used for this experiment (Fig. 2). Both pool 1 (oligo- and polynucleosomes) and pool 2 (mono- and dinucleosomes) absorbed RF from serum Jos, but not from serum Ter (Fig. 2). At saturation about 85% of RF Jos was removed; this required 0.6 µg of DNA from pool 1 and 150 µg of DNA from pool 2 (Fig. 2).

In this experiment it was possible that serum IgG bound to nucleosomes and that RF reacted with bound IgG rather than with the nucleosomes themselves. IgM from serum Jos was therefore prepared (see legend to Fig. 2). A solid phase radioimmunoassay using immunospecifically purified ¹²⁵I-labelled rabbit anti-human H(γ) chain antibodies showed that the IgM preparation contained only 0.6% IgG. Nevertheless, the two nucleosome pools absorbed the same proportion (~85% at saturation) of RF from IgM as from diluted serum (Fig. 2). As the amount of IgM used was 11.5 µg, only 69 ng (0.6%) of IgG was present. From earlier studies it is known that about 25% of IgM in serum Jos is RF⁶. This means that 2,400 ng (2.6 × 10⁻¹² mol) of IgM–RF would have to be absorbed by 69 ng (0.46 × 10⁻¹² mol) of IgG, assuming that all IgG were bound to nucleosomes and thereby rendered insoluble in 150 mM NaCl. Both these events are very unlikely, and it is therefore concluded that RF Jos do indeed bind directly to nucleosomes. Further tests supported this conclusion, as nucleosomes (pool 2) absorbed 82% of RF Jos isolated immunospecifically by elution from human IgG insolubilised on beaded Sepharose. Thus, the same proportion of RF was removed from serum, the IgM and the isolated RF fraction.

To determine whether the antigenic determinant on nucleosomes which was recognised by RF Jos required the presence of histones H1 and H5, polynucleosomes stripped of H1 and H5 were prepared (see Fig. 1b and legend to Fig. 1). This material (150 µg as DNA) which was soluble in phosphate-buffered saline (PBS), displaced 87% and 77% of RF derived from serum and IgM Jos, respectively, but none of the RF from serum Ter (Table 1). By contrast, when 1,180 µg of deproteinised double-stranded calf thymus DNA with an average size of 160 base pairs was mixed with IgM Jos (5.55 µg), only 20% of RF was displaced; this value is at the significance limit for the assay. Evidently, the determinant for crossreacting RF Jos is present on the nucleosome core which consists of DNA plus H2a, H2b, H3 and H4.

The polynucleosomes of pool 1 absorbed no anti-blood-group I activity from an isolated monoclonal IgM(κ) cold agglutinin (data not shown). This result, together with their lack of binding of RF from serum Ter, supported the conclusion that RF Jos reacted specifically with the nucleosomes.

The radioimmunoassay for RF depended on the reaction of labelled rabbit anti-human L-chain antibody with RF bound to

IgG on the tube wall. It could be argued that the labelled rabbit antibodies did not bind to RF with their active sites but with their Fc regions, which might function as an antigen for RF bound on the tube wall. However, addition of a 1,000-fold excess of nonspecific 'cold' rabbit IgG in the second step of the reaction decreased binding of ^{125}I -anti-L-chain antibody by only 5% which ruled out this objection.

The present results confirm that RF Jos recognise a nuclear antigen in addition to IgG, and establish that the majority (85%) of these polyclonal RF belong to the crossreacting population. Furthermore, the data indicate that the nuclear antigen is found on a complex consisting of DNA and the four core histones H2a, H2b, H3 and H4. This conclusion is based on the following observations. About 2,400 ng of IgM RF (2.6×10^{-12} mol) was absorbed by only 600 ng (with respect to DNA) of nucleosome pool 1 (Fig. 2); this corresponds to 6×10^{-12} mol of DNA with a size of 200 base pairs, which is the DNA content in the monomeric nucleosome¹². This nucleosome fraction contained only a trace of non-histone proteins (Fig. 1e), it still bound RF after H1 and H5 had been removed (Table 1), and RF Jos did not bind to native DNA alone⁶.

The structural basis for this remarkable crossreaction is still unclear. There has been no report showing homology between sequences of the Fc(γ) region and any of the existing histones, although some similarity has been proposed between a phylogenetically ancient H4 peptide and certain V-region sequences shared by human κ and λ Ig L-chains¹³.

However, as human L-chains do not displace RF Jos from nuclei while Fc(γ) does⁶, this homology cannot explain the

Table 1 H1+H5 stripped polynucleosomes bear antigenic determinant for rheumatoid factors (RF) in serum Jos

Antibody	H1+H5 stripped nucleosomes*	RF bound (c.p.m.)	RF displaced† (%)
Serum Jos 1/800	—	21,899	—
Serum Jos 1/800	+	13,366	87.5
IgM Jos 11.5 μg	—	13,931	—
IgM Jos 11.5 μg	+	9,876	77
Serum Ter 1/200	—	15,117	—
Serum Ter 1/200	+	15,411	0
Normal serum 1/200	—	3,000	—

0.15 ml (150 μg) of H1+H5 stripped polynucleosomes in PBS, pH 7.0, was mixed with 1.1 ml antibody dilution in PBS containing 1% BSA, and incubated for 2.5 h at room temperature. The mixture was then assayed for RF activity, and the % displacement calculated as described in the legend to Fig. 2.

* Chromatin fragments subjected to gel filtration in 0.6 M NaCl (see legend to Fig. 1).

† Based on comparison of c.p.m. bound by dilutions of the RF preparation in the absence of inhibitor (see legend to Fig. 2 for RF assay).

crossreaction. It is also unlikely that the determinant is a carbohydrate, as histones are not glycosylated. Possibly the two determinants recognised by the polyclonal RF Jos do not display that close structural similarity which past experience with cross-reacting antigens would lead one to expect. This is apparently the case for the polyclonal rabbit antibodies induced by immunisation with the hapten dinitrophenol, a large proportion of which crossreacts with menadione (vitamin K3)¹⁴; in this instance, the structural similarity between the two haptens is

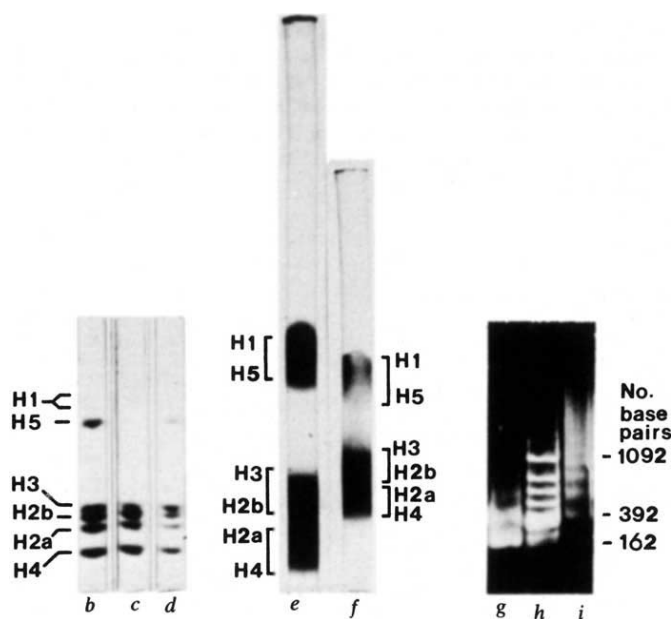
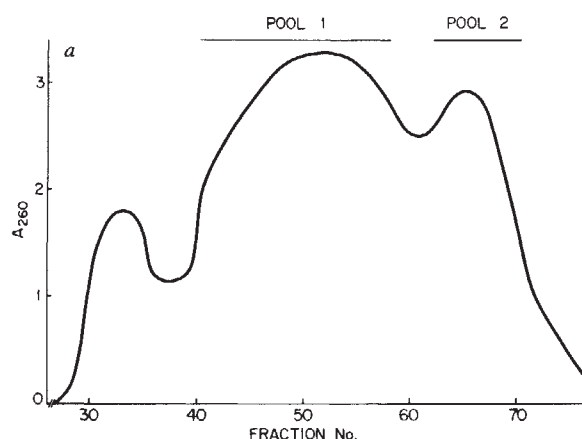


Fig. 1 Sepharose 4B gel filtration of chromatin extracted from micrococcal nuclease-digested chicken red cell nuclei. Adult chicken red cells were washed three times and nuclei prepared according to Shaw *et al.*⁷. The nuclei were resuspended in digestion buffer (0.3 M sucrose, 0.01 M Tris-HCl, 0.75 mM CaCl_2 , pH 7.2) to give $A_{260} = 70$ (read as diluted in 0.1 M NaOH) and digested for 10 min at 37°C with 20 units of micrococcal nuclease (Worthington) per 24 A_{260} units. The digestion was terminated by addition of disodium EDTA to a final concentration of 0.01 M and chilling samples in an ice bath. The suspension was centrifuged at 12,000g for 15 min. The pellet was resuspended in 0.2 mM EDTA, pH 7.0, incubated overnight at 4°C and centrifuged at 10,000g for 10 min. The extracted chromatin fragments of the supernatant were subjected to gel filtration on Sepharose 4B (7 ml supernatant containing 455 A_{260} units) on a 2.5×75 cm column equilibrated with 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.4. The fractions (5 ml) were pooled as indicated by the horizontal bars and concentrated by negative pressure filtration. 96% of pool 1 was insoluble in 150 mM NaCl. Of pool 2, 60% was insoluble in 150 mM NaCl, and only this fraction was used; it was redissolved in 2 mM EDTA and dialysed against 0.2 mM EDTA, pH 7.0. Another 7-ml aliquot of the supernatant was fractionated on the same Sepharose 4B column, now equilibrated with 0.6 M NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.4. The elution profile (a) was almost identical to that shown with the low ionic strength buffer; fractions corresponding to pool 1 were dialysed against PBS. DNA from the various pools was extracted with an equal volume of redistilled phenol saturated with H_2O . b, c and d show the histone composition of pool 1 (b), pool 1 from 0.6 M NaCl gel filtration (c), and 150 mM NaCl insoluble pool 2 (d). Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was carried out⁸. e, f show the purity of pool 1 (e) and 150 mM NaCl-insoluble pool 2 (f) analysed on SDS-7.5% polyacrylamide gels⁹ loaded with 60 μg (with respect to DNA) sample and stained with Coomassie blue; g, h, i show DNA fragment sizes of pool 1 (g), *Hinc*II digest of DNA from phage X174 (New England Biolabs) (h) and 150 mM NaCl-insoluble pool 2 (i). Electrophoresis of DNA was carried out in 1.4% agarose slab gels¹⁰ containing 40 mM Tris, 5 mM acetate, 1 mM EDTA, pH 7.9, and DNA bands were stained with ethidium bromide 1 $\mu\text{g ml}^{-1}$.

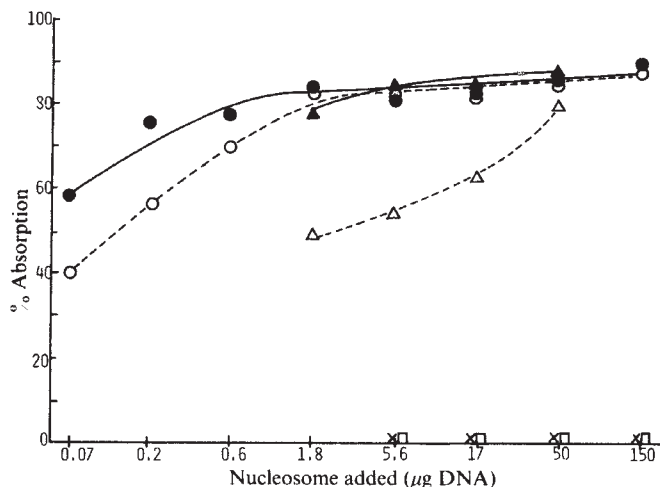


Fig. 2 Specific absorption of rheumatoid factor (RF) from serum IgG and IgM by nucleosomes. IgM was prepared by gel filtration of serum on Sephadex G-200 equilibrated in 0.5 M NaCl, 0.2 M acetate, pH 4.0; the first three fractions of the breakthrough peak were pooled, and dialysed against PBS pH 7.2. Absorption of RF was effected by mixing the indicated amount of nucleosomes in 80 μ l of 0.2 mM EDTA, pH 7.0, with 1 ml of serum or IgM diluted in PBS containing 1% bovine serum albumin (PBSA). The solution turned slightly turbid immediately on addition of the nucleosomes. After incubation at room temperature for 2.5 h, the insoluble nucleosomes were sedimented by centrifugation. The supernatants were assayed for RF activity by a solid phase radioimmunoassay¹¹ using plastic tubes (Falcon 2052) precoated with rabbit IgG. The second antibody was immunoaffinity isolated ¹²⁵I-labelled rabbit anti-human L-chain antibodies. The incubation time for the first and the second antibody was extended to 18–24 h. The amount of RF absorbed by the nucleosomes was determined from standard curves obtained by making twofold dilutions of the RF-containing sample in PBSA. Absorption of 75% of RF thus corresponds to the radioactive antibody bound by a standard sample diluted fourfold. $\circ$, serum IgG diluted 1/800 + nucleosome pool 1; Δ , serum IgG 1/800 + pool 2; $\bullet$, IgM Jos (11.5 μ g) + pool 1; $\blacktriangle$, IgM Jos (11.5 μ g) + pool 2; $\square$, serum Ter 1/200 + pool 1; $\times$, serum Ter 1/200 + pool 2.

limited¹⁵ and hydrophobicity may be an essential shared component. A few homogeneous myeloma proteins and Waldenstrom macroglobulins with antigen-binding activity have also been found to possess more than one specificity^{15–18}. Thus, RF Jos and similar RF detected in other sera^{5,6} may be examples of 'polyfunctional' antibodies.

The crossreacting RF described in the present report emphasises the general problem of extrapolating from the specificity of natural antibodies to the original antigenic stimulus which elicited their production. Moreover, the observations indicate that caution should be exercised in the interpretation of test results where RF are used as reagents, for example in detection of immune complexes.

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Hydrophobic character of amino acid residues in globular proteins

PREDICTIVE studies on the secondary structure of globular proteins aimed at locating ordered structural segments have provided little information about spatial orientations or even as to whether residues are exposed or buried. However, the physico-chemical properties¹ of residues can be used to obtain such information. In particular the hydrophobic character², is a useful parameter in these studies. The hydrophobic character as defined by the indices given by Tanford³ and Jones⁴ does not reflect hydrophobic environment within protein structures, but we introduce here a new parameter, the 'bulk hydrophobic character' obtained from an analysis of the surrounding hydrophobic environment of amino acid residues in protein crystals. This is a better index of protein hydrophobicity, showing very good correlation with the extent to which residues are buried⁵ (correlation coefficient $r = 0.9$) compared with the hydrophobic indices used previously, and it could be used to characterise tertiary structures.

Our study is based on crystallographic data on 14 protein molecules (myoglobin, ribonuclease S, cytochrome *c*, lysozyme, staphylococcal nuclease, carboxypeptidase A, subtilisin BPN', α -chymotrypsin, carp myogen, cytochrome *b*₅, apolactate dehydrogenase, trypsin inhibitor complex, concanavalin A and flavodoxin; see ref. 6 for further details on these proteins). They include representative examples of all known characteristic structural types of proteins defined by Levitt and Chothia⁷.

We demonstrated previously⁶ that a sphere of 8 Å radius has the required volume for any one residue of a protein molecule for a study of the influence of the surrounding environment. In this report, therefore, we define the 'surrounding hydrophobicity' of a residue as the sum of the hydrophobic indices assigned to the various residues that appear within an 8 Å radius volume in the protein crystal. This parameter is given by:

$$H_j = \sum_{i=1}^{20} L_{i,j} h_i$$

where $L_{i,j}$ is the total number of surrounding residues of the i th type associated with the j th residue in a given protein, and h_i is the hydrophobicity index for the i th residue. All the H values that belong to the same type of residue in the 14 crystals have been grouped together, and the arithmetic average ($\bar{H}$) for each type has been calculated. These average surrounding hydrophobicities are given in Table 1, together with the corresponding individual hydrophobic indices given by Tanford³ and Jones⁴. These ($\bar{H}$) values reflect the preferred hydrophobic environments for the residues and hence their 'bulk hydrophobic character'.

Table 1 Average surrounding hydrophobicities and the hydrophobicity indices for 20 amino acid residues occurring in globular proteins

Residue	Average surrounding hydrophobicity ($\langle H \rangle$) (kcal)	Hydrophobicity index (h_i) (kcal)
Ala	12.97	0.87
Asp	10.85	0.66
Cys	14.63	1.52
Glu	11.89	0.67
Phe	14.00	2.87
Gly	12.43	0.10
His	12.16	0.87
Ile	15.67	3.15
Lys	11.36	1.64
Leu	14.90	2.17
Met	14.39	1.67
Asn	11.42	0.09
Pro	11.37	2.77
Gln	11.76	0.00
Arg	11.72	0.85
Ser	11.23	0.07
Thr	11.69	0.07
Val	15.71	1.87
Trp	13.93	3.77
Tyr	13.42	2.67

In Fig. 1 the individual hydrophobic index (heavy line) is compared with the corresponding preferred surrounding hydrophobicity (thin line) standardised with respect to Gln. Both Fig. 1 and Table 1 show that the surrounding hydrophobicity is not proportional to the corresponding hydrophobic index of the residue. Taking the actual values given in Table 1, the residues can be divided into three groups: Cys, Phe, Ile, Leu, Met, and Val with $\langle H \rangle$ values greater than 14.0 kcal belong to group I. Gly, Ala, His, Trp and Tyr fall into group II with $\langle H \rangle$ values between 12.0 and 14.0 and are thus less hydrophobic than group I residues, and the remaining residues Asp, Glu, Lys, Asn, Pro, Gln, Arg, Ser and Thr with values below 12.0 kcal belong to group III. Val and Ile have the highest surrounding hydrophobicity, with Leu, Cys and Met coming next in order. Though Val is less nonpolar than residues such as Ile, Phe and Trp when defined by thermodynamic transfer measurements according to Tanford, the average surrounding hydrophobicity value suggests that this residue can easily be accommodated inside the molecule. The low value of $\langle H \rangle$ for Trp and Tyr indicate that one polar atom even in a large nonpolar side chain is sufficient to cause a large reduction in the hydrophobic environment. When considering the residues in group III, it is important to note that the surrounding hydrophobicity of Pro lies within the range observed for the polar residues. As the characteristic structure of proline does not allow it to occur in

either β strands or helices except at the N terminus, it is restricted to loops that inevitably lie on the surface thus lowering the surrounding hydrophobicity. Of the polar residues Gln, Glu and Arg have higher hydrophobicities, and the preferred environments for the charged residues Asp and Lys are associated with the lowest surrounding hydrophobicities.

Our results show that Val with an hydrophobic index almost half that of Trp, has the highest surrounding hydrophobicity. On the other hand, the surrounding hydrophobicity of Trp is very much reduced even though this residue appears at the top of the hydrophobicity index scale. The same conditions prevail for residues Tyr and Pro which, in spite of high hydrophobic indices, exhibit surrounding hydrophobicities similar to those of more polar residues. These observations suggest that apart from the nonpolarity indicated by the hydrophobic indices of amino acid residue side chains, both their structure and the presence of some polar atoms strongly influence their bulk hydrophobic character and hence their spatial position in protein molecules. Surfaces of globular proteins often contain several nonpolar residues^{1,8}, mainly group II residues. In our earlier report⁹ we pointed out that surface nonpolar residues occur as short distorted α -helical and extended structural domains (one to three residues long) with the backbone polar atoms having high solvent accessibility. The $\langle H \rangle$ parameter therefore characterises the hydrophobic environment preferred by each amino acid residue in a globular protein and we suggest that this specific preference for each residue is an important factor in the folding of a linear chain into a globular shape. We are now studying applications of this parameter to protein folding.

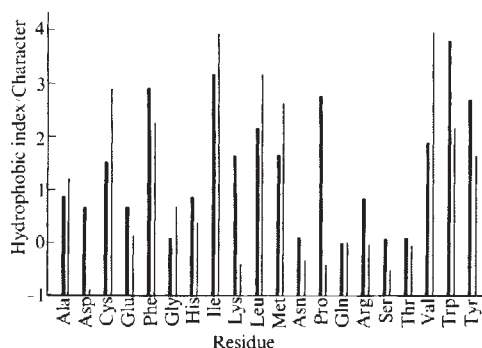
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Fig. 1 Comparison of the average bulk hydrophobicity of amino acid residues with their corresponding hydrophobic indices: thin line, average hydrophobicity; broad line, hydrophobic index.

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reviews

Fenland ecology

Peter D. Moore

Fenland: Its Ancient Past and Uncertain Future. By Sir Harry Godwin. Pp. 196. (Cambridge University Press: London and Cambridge, 1978.) £7.95.

THERE is no better living demonstration of the interaction of man with the natural environment anywhere in Britain than in the fenlands of East Anglia. Throughout prehistory and history these extensive tracts of swamp-land and bog have proved both a limitation on human mobility and settlement and, in more recent times, a valuable agricultural resource. A number of books, especially those of H. C. Darby, have considered man's conflict and symbiosis with fenland, particularly from the historical geography point of view, but this book approaches the subject from the biological angle.

In this sphere, Sir Harry Godwin is the ideal person to write such a book, for the story of the unravelling of fenland's history is essentially Godwin's autobiography. Indeed, the book has a strongly autobiographical, almost anecdotal style. It is written in an informal manner for a general readership; it avoids technical terms and jargon and does not carry references in the text, although a reading list is given at the end. This makes it easy, even exciting reading, which should prove attractive to students.

One advantage of the relaxed style is that we are provided with some rare glimpses into those chance events in history which have profoundly influenced the development of ecology in this country. Take, for example, that priceless sentence which introduces the chapter on pollen analysis, "In 1931, following a suggestion from A. G. Tansley, my wife began research in pollen analysis, a technique recently brought to notice by the Swede G. E. Erdtman . . .". It is difficult now to assess the full consequences of Tansley's chance remark.

Much of the book is concerned with the stratigraphy and palaeoecology of the fenland sediments. Rooted in the basal clays of the fens at many sites were found the preserved remains of giant bog oaks, many having dimensions far exceeding those found commonly in modern British forests; often they were over 27 metres in height.

These were inundated and killed by rising water tables as the true fens were formed. Subsequently, further woodland of a swampy nature developed over the reed bed sediments, and the sequence of species in these fen woods, leading ultimately to the establishment of pine, is of particular interest, for one finds similar developments on the other side of the North Sea and in some of the west coast submerged forests, as at Borth and Ynyslas, Dyfed.

The archaeological interest of the fenland peats is again introduced by a personal reminiscence: "I recall picking out (from the borer) what I took to be a bit of pine bark: licking it clean of peat . . . it shewed itself to be a struck flake of flint." Thus began the excavations of Shippea Hill and Peacock's Farm in fenland, now classic archaeological sites.

One of the most interesting questions in peatland ecology raised by Godwin's fen studies, is the extent to which true raised bog communities once flourished in the east of Britain. Peat cutting and agricultural developments have destroyed much of the evidence of past acidification of the fen systems leading to bog formation. At Woodwalton Fen, Godwin describes the surviving stratigraphic evidence for this process and at this site there still remain some frag-

ments of a relict, acidophilic community on the surface.

The latter part of the book concentrates on the hand of man in the modification of the fenland environment, and on the habitats and species lost as a result of exploitation. Perhaps the most important message of this book as far as mire conservation is concerned is the vulnerability of the ombrotrophic, raised mire system. The loss of this habitat in eastern England cannot be repaired; such a late stage in mire succession takes millennia to develop and the lesson of the fens should serve as a warning to those concerned with mire conservation in northern and western Britain, where similar exploitation is now occurring and where similar consequences are to be feared.

This book is essentially a compilation of scattered, largely previously published material, which is presented in a lucid, informal and readable manner. It is not so much a textbook as a personal view of a distinctive part of Britain and of the development of a field of environmental research in which the author has himself played the leading role. □

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Mathematical models in immunology

Theoretical Immunology. Edited by G. I. Bell, A. S. Perelson and G. H. Pimbley, Jr. (Marcel Dekker: New York and Basel, 1978.) Sfr. 128.

GEORGE BELL'S mass action model of the immune response made it respectable to use computer simulation in immunology. Now eight years later we have an opportunity to see how the field has progressed. This volume provides the first way of doing this conveniently, and as such can be recommended to professionals and general students. Although multi-authored, it is not a miscellaneous collection, but has a logical sequence with useful introduction and indexes. Several of the papers come from Bell's

own group of renegade theoretical physicists at Los Alamos.

As judged from this collection, mathematical models contribute more to immunology than to, say, embryology, and less than to fisheries research. Reasonable assumptions about lymphocyte triggering and regulation generate models which fit well empirical data on affinity maturation, high and low zone tolerance, and cycling antibody production. The models seem to be flexible to an extent which augurs well for the future as new phenomena are discovered, but which makes one a little uneasy at present. Thus, some models run quite satisfactorily without network suppression (Bruni, Mohler), whereas others depend on this entirely (Richter, Hoffman). Another difficulty is that in their latest version as expounded here, some of the models have grown mathematically rather

baroque.

A second group of theories deal with useful treatments of the MIF and Jerne plaque assays, the latter showing that several assumptions commonly made by experimentalists are probably wrong. Some common cook-book analysis—for example, of antibody affinity—is omitted. A third group deal with local phenomena: B cell triggering, cell recruitment (including a particularly elegant treatment of lymphocyte traffic), and antibody fit. Inman's handling of the antibody frequency relationship improves on but

omits reference to my earlier work.

The weakest part comes from the experimentalists, who should have provided balanced reviews. Dual recognition is not as obscure as Burnet and Dutton make out, but does provide a problem for helper factors which Feldmann skips. The networkers have yet to take up Raff's challenge (*Nature*, 265, 205; 1977).

N. A. Mitchison

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Barley handbook

Barley. By D. E. Briggs. Pp. 612. (Chapman and Hall: London, 1978.) £25.

BARLEY is one of those crops, like wheat or cotton, with a fascinating biology and a complicated technology to match. Dennis Briggs makes a valiant attempt at a monographic treatment of all aspects of this, the world's fourth most important cereal crop. His book is a useful addition to the literature, although almost inevitably it suffers from unevenness.

The book begins with chapters on the morphology of the vegetative and reproductive plant. These are welcome, although it is unfortunate that the relevance and quality of some of the diagrams, often taken from rather elderly works, is doubtful; a page of illustrations of types of hood found in barley seems excessive, and better drawings, or even scanning electron micrographs, of the developing ear would have been valuable. The origin and classification of barley is discussed in a brief chapter and barley genetics are considered as a prelude to a chapter on crop improvement. To a non-specialist the treatment seems rather superficial, but a great deal of the recent literature is quoted, and this is a feature of the book as a whole.

Three chapters on barley biochemistry, grain quality and germination, and growth of the barley plant occupy about a third of the book. They are informative and give a comprehensive picture of almost every aspect of barley development and metabolism, at the price of being rather unselective and presenting biochemical pathways in somewhat exhaustive detail.

Agricultural aspects of the barley crop take up five chapters. That on agricultural practices and yield is brief and on occasions rather trivial. It is complemented by chapters on weeds, pests and diseases, and problems of

storage. Here one looks in vain for data on estimates of crop losses due to plant and insect pests, but perhaps in consolation we are given a formidable list of diseases carried by the rat. This rather unsatisfactory section also includes a chapter on production and harvesting machinery which is of doubtful value. No-one is going to go

Diatomic molecules

Diatomic Molecules: Results of Ab Initio Calculations. By R. S. Mulliken and W. C. Ermler. Pp. 197. (Academic: New York, San Francisco and London, 1978.) \$19.50.

It seems churlish to greet any publication which has Robert Mulliken as an author with something less than total enthusiasm. His contributions to molecular physics have been truly monumental and his personality has inspired affection in those who know the man and his work. The choice of topic for this book again raises high expectations.

Diatomic molecules have always been wonderful model systems on which to base and test theories. Their spectroscopy is so rich and the results are so accurate and unambiguous that they have always attracted high calibre experimentalists as well as theoreticians. Nor is their importance declining: far from it, as the advent of lasers has provided tools for even more subtle experiments. The diatomic molecule has been the subject of vast numbers of experiments and its importance breaks out from the vicious circle of experiments being done for theoreticians who do calculations to explain experiments. Two-atom molecules are of wider interest, because of their key roles in interstellar molecule formation, atmospheric physics, energy transfer mechanisms and in the development of new laser systems.

The time is clearly ripe for a major

to this book to be informed on types of ploughs, drills or combine harvesters.

The final part of the work deals with the many uses of this crop. Here the author returns to surer ground and the chapter on barley as animal and human food contains many interesting facts. The mysteries of malting are made reasonably clear in a brief but direct chapter and the book ends with a survey of the uses of barley malt, including the making of vinegar and more potable liquids.

At the price few individuals are likely to purchase this book. However, despite its shortcomings it is a work which should find a place in all laboratories dealing with cereals. It is to be hoped, however, that library copies do not show the blurred print which marred parts of the review copy.

J. E. Dale

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work on all aspects of the physics and chemistry of diatomic molecules. This is not that book, but any such publication might well be dedicated to Robert S. Mulliken.

The actual work under review provides a survey and analysis of computational studies of the electronic structure of ground and excited states of diatomic molecules. It is neither a student text nor a full monograph. It commences with a useful summary of theory which can largely be found in a number of other texts and which is probably too condensed for the novice and too familiar for the expert. Succeeding chapters treat one-electron molecules, two- to four-electron molecules, hydrides and finally homopolar and heteropolar species.

Electron correlation is emphasised as are charge distributions, but the other molecular properties which are the object of *ab initio* calculation are only given a cursory treatment. The bibliography contains some 1976 references but in the text an important 1974 paper is described as 'recent'.

Perhaps the combination of distinguished authors and important topics raises expectations which are bound to be disappointed. The book will be welcomed by the small band of theoreticians who work on diatomic molecules but will probably miss the potentially wider audience. It might just provoke someone to write or edit the major tome on all aspects of diatomics.

Graham Richards

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obituary

G. W. Kenner, 1922–1978

WITH the tragic death of Professor G. W. Kenner at the early age of 55 we have lost a most distinguished organic chemist, and the leading authority on peptide and protein synthesis.

George Wallace Kenner was born in Sheffield in 1922, of parents who were both chemists; his father, James Kenner, F.R.S., became Professor of Technological Chemistry in Manchester in 1928 and George spent most of his boyhood there attending the Grammar School. Although he was educated and excelled on the classical side at school, the pull of the family profession was obviously strong for he turned to chemistry when he entered University in 1939. First in Manchester and then in Cambridge he displayed prodigious talents for science, still recalled by his contemporaries. Possibly because of the breadth of his education, his early interests in chemistry covered an unusually wide span and continued to do so for the rest of his career.

His first research, in Manchester with Professor (now Lord) Todd, was on nucleotide synthesis and this, together with the synthesis of nucleotide co-enzymes, continued when the group moved to Cambridge in 1944. The structure, chemistry, and total synthesis of biologically important compounds was to remain Kenner's consuming research interest.

In 1957, as possibly the brightest of Todd's galaxy of young Cambridge stars, he came to Liverpool to the Heath Harrison Chair of Organic Chemistry, in succession to a select band of organic chemists: Sir Robert Robinson (1915–20), Sir Ian Heilbron (1920–33), and Alexander Robertson (1933–57). He found a department which, though well-known as a centre for classical natural product research, was rather run down and was very deficient in modern equipment. Sheer ability and enthusiasm gained him the support of the staff and with them he quickly built up an impressively equipped modern department. Almost immediately he started to plan the splendid Robert Robinson Laboratories

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which, occupied in 1961, are still in the forefront of modern chemistry buildings and now provide a permanent monument to his life and work. Working in them nearly twenty years later, one can still sense the explosive energy at the origin of this particular part of the universe.

He showed vision and magnanimity in building up the department, in creating the right atmosphere for good research, and in the staff he appointed. Particularly imaginative was his invitation to Alan Battersby to a new Second Chair of Organic Chemistry in 1962, one of the first such appointments in the country. Their combined talents contributed greatly to organic chemistry and established the department as an international research centre which, in the twenty-one years of Kenner's leadership, expanded beyond recognition.

During this time he also played an important part in undergraduate teaching and in University affairs. While he served skilfully on many major boards and committees, he was not a committee man. He trusted more to the benign dictatorship of departmental

heads and provided a splendid example of that system at its best. He became steadily less tolerant of recent democratic trends in Universities and the associated bureaucracy; his Royal Society Research Professorship, which was the first ever held in Liverpool, brought a welcome relief from many administrative chores.

He was deeply dedicated to his work and expected much of his associates; indeed he was fond of equating academic freedom with the freedom to work still harder! Sadly, the standards he demanded of himself were often unattainable and he sometimes suffered, as perfectionists must. He always said and did exactly what he thought right, regardless of personal considerations and expediency. He was, nevertheless, a splendid colleague, charming and unfailingly courteous, lively and forceful, and there was rarely a dull moment in his company! He had a superb command of English, spoken and written, and his early classical training often erupted with flair, never more pungently than when dealing with sloppiness of thought or action, from whatever source. Even away from the laboratory George did not do things by halves, and he gained immense pleasure from sailing, hill walking, and driving, buying and selling fast motor cars and motor-bikes.

His scientific originality and judgement were widely respected and his services were constantly in demand by industrial, academic and professional organisations. He was a much valued industrial consultant and a champion of genuine collaboration between academic and industrial research, for which he greatly admired the Swiss. His extensive and important scientific work in Liverpool, especially in the fields of porphyrin and protein synthesis, culminated in the present herculean task of total synthesis of a protein closely related to the natural enzyme lysozyme, with the aim of shedding light on the mechanism of lysozyme action. His synthetic techniques and criteria of purity were those of the rigorous organic chemist since

he emphatically rejected the view that standards could be relaxed when the molecules became large and complex. The linear sequence of Liverpool lysozyme's 129 amino acids, fully protected, has been constructed and the final, though demanding, phase of the work is concerned with removal of the protecting groups. Although he will not see the conclusion of this pioneering work, he had already brought new scientific standards to the art of peptide synthesis and provided a firm foundation on which others can, and undoubtedly will, build in the future.

This work had brought him most honours and distinctions available to a British chemist. He was awarded the Meldola and Corday-Morgan Medals, gave the Tilden, Simonsen, and Pedler Lectures of the Chemical Society, and the Bakerian Lecture of the Royal Society. This last, *Towards Synthesis of Proteins* (*Proc. R. Soc. A* 1977, **353**, 441), provides a fascinating account of the strategy and achievements of the lysozyme project.

In spite of the constant demands upon him he always found time to help with the countless problems brought to him. He had an impressive memory for all who passed through the department and he followed their subsequent careers with genuine interest. Recent tragic events have shown just how many people respected and revered him, and are now left, with his wife and daughters, to mourn this splendid man.

C. W. Rees

secret ballot, Keldysh's appointment was largely seen abroad as being associated with expansion of the Soviet space programme.

As President of the Academy he played a major role in the expansion of Soviet science, and the development of a Union-wide network of scientific research establishments and centres. His speeches on the progress of Soviet science became a prominent feature of virtually all Party and academic occasions.

Keldysh retired from the Presidency of the Academy in 1974, shortly before the belated celebrations of its 250th anniversary. He still continued, however, to take an active part in the organisation of Soviet science, serving, for example on the Committee for Lenin and State Prizes.

For his services to Soviet science, Keldysh was honoured during his lifetime by the title of Hero of Socialist Labour (three times), Lenin and State prizes and numerous medals and orders of the Soviet Union and the countries of the socialist bloc. As a further and final tribute he was accorded a state funeral, his ashes being enshrined in the Kremlin wall.

Vera Rich

in the physiology department, including some to the students of the refugee Polish Medical Faculty in Edinburgh.

When Dr Daly was appointed first Director of the Agricultural Research Council Institute of Animal Physiology at Babraham, Cambridge, he invited Catherine Hebb to join his staff. Dr Hebb's work on the lung vasculature, together with a year's work with Professor W. Feldberg in Cambridge, during the tenure of a Senior Beit Fellowship, led her to an interest in the autonomic system and neurotransmitters, and it was in this field that her most important scientific contribution was made. She established at Babraham a first-rate neurochemical laboratory, and in 1965 became the head of the Institute's physiology department.

Chemical transmission from nerve-ending to effector organ had been familiar to physiologists for decades, but neuron-to-neuron transmission, in ganglia and in the central nervous system itself, was a newer, promising and technically difficult field when Dr Hebb began work in it. Her studies on the mechanism of the synthesis of acetylcholine, and of the distribution of the enzymes concerned in its metabolism, combined the techniques of biochemistry, histochemistry and classical pharmacology. However, among all this fine detail she never lost sight of the physiological functioning of the whole animal, and for this reason among others, she was able to write excellent review articles, putting her own discoveries and those of others in their proper context. The Physiological Society monograph on the vasculature of the lung which she wrote in collaboration with I. de Burgh Daly in 1966 is still a standard work.

Dr Hebb was notable for the sympathetic encouragement which she gave to younger workers in the field of neurochemistry, including a number from Poland, Yugoslavia, Czechoslovakia, Hungary and South American countries. She helped to bring such workers to Babraham for periods of research and study, thereby disseminating enthusiasm for the subject and also a wish to emulate her own high standards of work.

Dr Hebb had already been battling against ill-health for some ten years when she retired from Babraham in 1976. Her concern for others was typified by her request that the collection made to mark her retirement should be used to establish a loan fund to help staff, visiting scientists or students at Babraham in financial need. Contributions to this Catherine Hebb Loan Fund have flowed in from every continent—an indication of the width of the affection for Catherine Hebb and admiration for her work.

M. W. Stanier

M. V. Keldysh

MSTISLAV VSEVOLODOVICH KELDYSH, former President of the Academy of Sciences of the USSR, died on 24 June 1978.

Keldysh was born in Riga in 1911. He graduated from the Faculty of Physics and Mathematics in Moscow State University in 1931, and then went to the Zhukovskii Central Aerodynamics Institute, where he worked on mathematical aerodynamics, including the theory of unstable wing movements and flutter.

Although Keldysh was later to be acknowledged as having played a founding role in the establishment of post-war Soviet technology—computer mathematics, nuclear engineering and space research—he remained, to the outside world, relatively unknown, save as a mathematician, until 1961, when he became President of the Soviet Academy of Sciences. Although the Academy still elects its President by

C. O. Hebb

DR CATHERINE OLDING HEBB, formerly Deputy Chief Scientific Officer, A.R.C. Institute of Animal Physiology, Babraham, Cambridge, died at Cambridge on 26 August 1978, aged 67.

Catherine Hebb was born in Nova Scotia and educated at Dalhousie University, Halifax, Nova Scotia, and at McGill University, Montreal. Here in 1937 she gained her Ph.D. and carried out her first research, on the secretory activity of the digestive glands, under Professor B. P. Babkin.

In 1938, Dr Hebb came to Edinburgh University as a Junior Beit Memorial Fellow, in the Department of Physiology, under the late Professor I. de Burgh Daly. For the next fourteen years, in collaboration with Daly and other members of his very active department, Catherine Hebb produced a series of papers on the control of the blood supply to the lungs by neural and chemical processes. During the Second World War, she and Daly undertook research in the department at Edinburgh on behalf of the Air Ministry and the Ministry of Supply. Throughout this period, until 1952, Dr Hebb was giving lectures to undergraduates

announcements

Meetings

7 December, **Strategy for Nutrition Research**, London (Dr M. R. Turner, British Nutrition Foundation, 15 Belgrave Square, London SW1, UK).

9 January 1979, **Annual Histochemistry**, London (The Administrator, Royal Microscopical Society, 37/38 St Clements, Oxford, UK).

13–15 March, **Safety of Electrical Instrumentation in Potentially Explosive Atmospheres**, Chislehurst (Mrs R. G. Keiller, Sira Institute Ltd, South Hill, Chislehurst, Kent, UK).

26–30 March, **Quantitative Microscopy (including image analysis)**, Cambridge (The Administrator, Royal Microscopical Society, 37/38 St Clements, Oxford, UK).

26–28 March, **Microscopy of Amorphous Solids**, Cambridge (The Administrator, Royal Microscopical Society, 37–38 St Clements, Oxford, UK).

28–30 March, **Mechanical Properties of Materials at High Rates of Strain**, Oxford (Meetings Office, The Institute of Physics, 47 Belgrave Square, London SW1, UK).

2–6 April, **Principles of EM and Specialised Transmission EM**, Leeds (The Administrator, Royal Microscopical Society, 37/38 St Clements, Oxford, UK).

9–11 April, **Microscopy of Semi-conducting Materials**, Oxford (The Administrator, Royal Microscopical Society, 37/38 St Clements, Oxford, UK).

24–27 April, **5th International Conference on Invertebrate Tissue Culture**, Lucerne (E. Kurstak, Département de Microbiologie et Immunologie, Faculté de Médecine, Université de Montréal, C.P. 6128, Succ.A. Montréal, P.Q. Canada H3C 3JL).

29 April–5 May, **15th EUCHEM Conference on Stereochemistry**, Burgenstock (Prof. Sir Derek Barton, FRS, Institut de Chimie des Substances Naturelles, CNRS, F-91190 Gif-sur-Yvette, France).

9 May, **3rd Annual Electron Microscopy Meeting**, Aston (The Administrator, Royal Microscopical Society, 37/38 St Clements, Oxford, UK).

6–8 June, **Towards 2001—New Information Technology and its Future Impact**, Torquay (Mike Allen, Beecham Pharmaceuticals, Coldharbour Rd, The Pinnacles, Harlow, Essex, UK).

2–6 July, **Course on Introduction to Advanced Imaging Techniques for Biologists**, Leeds (The Administrator, Royal Microscopical Society, 37/38 St Clements, Oxford, UK).

8–14 July, **International Conference on Climate and History**, Norwich (Conference Secretary, Climatic Research Unit, School of Environmental Sciences, University of East Anglia, Norwich, UK).

9–13 July, **2nd International Conference on the Mechanisms of Reactions in Solution**, Kent (Dr John F. Gibson, The Chemical Society, Burlington House, London W1, UK).

9–13 July, **1st International Botanical Microscopy Meeting**, York (The Administrator, Royal Microscopical Society, 37/38 St Clements, Oxford, UK).

10–12 July, **Immunocytochemistry**, York (The Administrator, Royal Microscopical Society, 37/38 St Clements, Oxford, UK).

16–18 July, **Automation in Industrial and Clinical Chemistry**, London (Beverly Humphrey, Scientific Symposia Ltd, 33/35 Bowling Green Lane, London EC1, UK).

16–19 July, **Endocrinology**, London (Conference Secretary, RPMS Du Cane Rd, London W12, UK).

Meetings, Awards, Appointments, On the Move, and Person to Person are listed on the "announcements" pages of *Nature*.

Entries for any of these sections should be sent to: *Nature*, 4 Little Essex St, London WC2, UK. (There is no charge for this service.)

The previous list of meetings was in *Nature* of 12 October, page xvii.

24–27 July, **Video and Data Recording**, Southampton (Conference Secretariat, Institution of Electronic and Radio Engineers, 99 Gower St, London WC1, UK).

30 July–3 August, **7th AIRAPT International High Pressure Conference**, Le Creusot (Andre Kusubov, University of California, Lawrence Livermore Laboratory, California 94550).

13–17 August, **Electron Microscopy Society of America: 37th Annual Meeting**, San Antonio (Dr William H. Massover, Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912).

19–23 August, **3rd International Conference on the Chemistry and Uses of Molybdenum**, Michigan (Climax Molybdenum Co. Ltd, Imperial House, 1/3 Grosvenor Place, London SW1, UK).

20–24 August, **Artificial Intelligence**, Tokyo (Prof. Raj Reddy, Dept of Computer Science, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213).

20–25 August, **3rd International Conference on Surface and Colloid Science**, Stockholm (The Swedish Institute for Surface Chemistry, c/o Stockholm Convention Bureau, Strädvagen 7C, S-114 56 Stockholm, Sweden).

26–31 August, **4th International Symposium on Environmental Biogeochemistry**, Canberra (The Conference Secretary, Australia Academy of Science, PO Box 783, Canberra City, ACT 2601, Australia).

28 August–1 September, **Neurochemistry of the Retina**, Athens (Dr Nicolás G. Bazán, Instituto de Investigaciones Bioquímicas, Universidad Nacional del Sur-Conicet, Av. Alem 1253, 8000- Bahía Blanca, Argentina).

29–31 August, **IUPAC Symposium on Mycotoxins and Phycotoxins**, Lausanne (Prof. P. Krogh, Dept of Veterinary Microbiology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana 47907).

30–31 August, **Japan Conference on Polymers and Plastics**, Tokyo (Vijay Mohan Bhatnagar, Alena Enterprises of Canada, PO Box 1779, Cornwall, Ontario, Canada K6H 5V7).

Reports & Publications

UK & Ireland—September

Birth Impairments. Pp. 60. (London: Office of Health Economics, 162 Regent Street, W1, 1978.) 35p. [19]

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The Radiochemical Centre Limited, Amersham. Seventh Annual Report for the year ended 31st March 1978. Pp. 20. (Amersham: The Radiochemical Centre Limited, 1978.) [19]

The Universities Central Council on Admissions. Statistical Supplement to the Fifteenth Report, 1976/1977. Pp. 28. (Cheltenham: The Universities Central Council on Admissions, PO Box 28, 1978.) £1.25 [19]

The Labour Party. Statements to Annual Conference. By the National Executive Committee. Pp. 67. (London: The Labour Party, Smith Square, SW1, 1978.) 45p. [219]

The Pattern and Range of Services for Problem Drinkers. Report by the Advisory Committee on Alcoholism. Pp. iii+52. (London: Department of Health and Social Security, SH Division, Alexander Fleming House, SE1, 1978.) gratis. [219]

Royal Commission on the National Health Service. Research Paper No. 4: Doctor Manpower 1975–2008—Alternative Forecasts and their Resource Implications. Pp. xiii+60. (London: HMSO, 1978.) £1.50 net. [219]

Philosophical Transactions of the Royal Society of London. A: Mathematical and Physical Sciences. Vol. 290, No. 1367: Atmospheric Chemistry—Response to Human Influence. By Jennifer A. Logan, M. J. Prather, S. C. Wofsy and M. B. McElroy. Pp. 187–234. (London: The Royal Society, 1978.) UK £3.15; Overseas £3.30. [229]

Tetrahedron Reports. No. 41: Bio-mimetic Oxygenation. By Teruo Matsuura. Pp. 39. No. 42: Structure and Reactivity of Alkali Metal Enolates. By Lloyd M. Jackman and Barry C. Lange. Pp. 35. No. 44: Action de l'Hydroxylamine, de l'Hydrazine et de ses Derivés sur les Pyrones. Par Christophe Morin et René Beugelmans. Pp. 13. No. 45: Applications Récentes de la Réaction de Retro-Diels-Alder en Synthèse Organique. Par Jean Ripoll et Annick Rouessac et Francis Rouessac. Pp. 25. No. 46: The Synthesis of Macrocyclic Lactones—Approaches to Complex Macrolide Antibiotics. By Thomas G. Back. Pp. 22. No. 47: The Problem of Conformational Effects. By N. S. Zefirov. Pp. 13. No. 48: Mesomeric Betaine Derivatives of Heteropentalenones. By Christopher A. Ramsden. Pp. 33. No. 49: The Biosynthesis of Aromatic Hemiterpenes. By Michael F. Grundon. Pp. 22. (Oxford and New York: Pergamon Press, 1978.) £4.95 each. [229]

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The Health Effects of Lead on Children: A Review of Literature Published Since 1976. Pp. 28. (Chertsey, Surrey: The Conservation Society, 12a Guildford Street, 1978.) £1. [229]

Water Research Centre. Annual Report, 1977/1978. Pp. 107. (Henley-on-Thames: Water Research Centre, 45 Station Road, 1978.) [229]

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Reflections on Atomic Energy History. By Margaret Gowing. (The Rede Lecture 1978.) Pp. 26. (Cambridge and London: Cambridge University Press, 1978.) 75p. [269]

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Engineering Seismology. By Professor N. N. Ambraseys. (Inaugural Lecture, 18 November 1975.) Pp. 53–60. £1.75. Some Images of Optics. By Professor W. T. Welford. (Inaugural Lecture, 27 January 1976.) Pp. 45–51+4 plates. £1. Cow Green Revisited. By Professor J. L. Knill. (Inaugural Lecture, 10 February 1976.) Pp. 61–71. £2. Splendours and Miseries of Concrete Structures. By Professor A. J. Harris. (Inaugural Lecture, 24 February 1976.) Pp. 73–82. £2. (London: Imperial College of Science and Technology, 1977 and 1978.) [299]

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